# SPERMATOGENESIS OF *DROSOPHILA HYDEI*: CHROMATIN REORGANIZATION AND HISTONE VARIANTS

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*ter herinnering aan mijn vader*

*voor mijn moeder*

CONTENT

Chapter I

# Introduction

DNA in the cell nucleus of eukaryotes, is organized together with RNA and proteins in a complex structure which is called chromatin.

The first protein typically associated with chromatin was described in 1884 by Albert Kossel, who described a peptone-like component isolated from goose erythrocyte nuclei by acid extraction (Kossel 1884). He named this component histone.

Histones have subsequently been recognized as universal and basic structural proteins of eukaryotic chromosomes. More than a century of research has brought a great deal of information about the structure of these proteins. Their function has been attributed to the organization of the DNA in the chromosome. They are responsible for the first level of packaging of the DNA within the nucleus by forming nucleosomes. As a consequence of this function in packaging, histones must have an influence on metabolic processes at the DNA level, such as replication and transcription. The main discussion has, therefore, always been directed towards a potential role of histones in regulating gene expression. However, histones seem also to be involved in other nuclear processes. For example, H1 can act as a recombinase that catalyzes ATP-independent DNA strand transfer (Kawasaki *et al.* 1989) and histones H2A and H2B were discussed as components of the homeostatic thymus hormone in calf (Reichhart *et al.* 1985).

This chapter summarizes histone research. It will be confined to an analysis of animal histones since information on plant histones is still less extensive and only recently emerging (for review see Chaubet *et al.* 1987; Wells and McBride 1989).

### *Classification of histones*

Histones comprise small basic proteins which are highly conserved during evolution (for reviews see Elgin and Weintraub 1975; Isenberg 1979; Wells and McBride 1989). On basis of their amino acid composition these proteins can be divided into five classes: the four core histones H2A, H2B, H3 and H4 as the basic constituents of the nucleosome core, and histone H1, which interacts with the linker DNA between adjacent nucleosome cores. The binding of H1 in chromatin is cooperative (Elgin and Weintraub 1975, table 1). H1 seems not to be present in yeast.

*H1.* Histone H1 is the largest molecule among the histones and shows the highest degree of primary sequence divergence both within and between species. The proteins of this class are very lysine-rich with an unequal distribution of the basic residues. Both amino (N) and carboxyl (C) termini are highly basic and rich in alanines and prolines and display a particularly high degree of sequence variation. The internal globular region of the proteins, which includes approximately 80 amino acids is less basic and is more conserved during evolution (Hartman *et al.* 1977; Böhm and Mitchell 1985; Clore *et al.* 1987; for review see Elgin and Weintraub 1975; Isenberg 1979; von Holt 1979; Cole 1984).

The precise location of H1 within the chromatin is not yet clear (*cf.* Mirzabekov *et al.* 1989). Evidence is accumulating that the different structural domains of H1 have different roles in chromatin structuring. The globular re-

gion of H1 is involved in the sealing of the two turns of DNA around the nucleosome (Thoma *et al.* 1979, 1983; Allan *et al.* 1980). This domain together with the C-terminal domain is capable of converting the chain of nucleosomes into a 30 nm fibril (Allan *et al.* 1986). A possible binding area for the C-terminal domain is the linker DNA between the nucleosomes (Rodriguez *et al.* 1991 and references therein). The role of the N-terminal domain of histone H1 is sofar rather obscure. It might be involved in protein-protein interactions in the higher order chromatin organization (Mirzabekov *et al.* 1989).

*H2A.* Among the core histones H2A shows the highest degree of divergence in its amino acid sequences. The degree of sequence conservation does not vary much along the protein, with the exception of a highly conserved stretch of nine amino acids (AGLQFPVGR) at the N-terminal end (see Wells and McBride 1989).

H2A proteins are lysine rich. As is true for histone H1, there is a nonuniform distribution of basic amino acids in proteins of the H2A class. The N-terminal third of histone H2A is more basic, whereas the C-terminal two thirds of the protein in their amino acid composition are as typical for globular proteins. This bipartite structure generally applies also to the histones H2B, H3 and H4 (for review see McGhee and Felsenfeld 1980; Wu *et al* 1986).

*H2B.* Also the members of this histone class are lysine-rich. The class of H2B proteins, in general is more conserved during evolution than those of H2A and H1. The proteins are evolutionary chimeres: the lysine- and arginine-rich N-terminal regions are highly divergent, whereas the hydrophobic globular C-terminal domains, comprising two thirds of the molecules, are highly conserved (reviewed by McGhee and Felsenfeld 1980; Wu *et al.* 1986).

*H3.* Histones belonging to the class of H3 show a very high degree of primary sequence conservation including both the C-terminal and the basic N-terminal region. Only the primary sequences of histone H3 from protists and fungi show a considerably higher degree of divergence (Wu *et al.* 1986). The basic amino acids consist for more than 50% of arginines.

The carboxyl terminal region of H3 is involved in interactions with the other core histones, an interaction which is probably responsible for the conservation of this part of the histone H3. The evolutionary pressure on the N-terminal region of H3 must be due to other protein interactions, which are so far unknown (reviewed by McGhee and Felsenfeld 1980; Wu *et al.* 1986).

*H4.* Histone H4 is the most conserved class of histones. H4 of calf thymus differs only by two conservative amino acids substitutions from H4 of peas (DeLange *et al.* 1969). Differences are more pronounced in the internal region than in the N- and C-terminal portions of the proteins. The globular C-terminal region of H4 is involved in histone-histone interactions which again might explain the particularly high degree of sequence conservation. As in H3 the divergence in fungi and protists is higher than between higher eukaryotes.

It has been mentioned above that the four core histones contain two distinct domains, a globular hydrophobic core and a hydrophilic N-terminal

tail with an increased number of positively charged amino acids. The globular part is involved in both histone-histone interactions and in histone-DNA binding (reviewed by McGhee and Felsenfeld 1980; Wu *et al.* 1986).

The function of the N-terminal tails of the core histones is less well understood. The tails are not essential for nucleosome formation. However, they might be involved in stabilization of the nucleosomes and in controlling an additional folding and unfolding of the chromatin (Encontre and Parello 1988; Ausio *et al.* 1989; for review see McGhee and Felsenfeld 1980; Wu *et al.* 1986). The N-terminal tail of H4 remained almost invariant in evolution. Deletion of this region in yeast H4 has a more pronounced effect on the cell-cycle and chromatin structure in comparison to deletion of this region in H2A or H2B which might explain the high conservation of the H4 N-terminal tail (Norris *et al.* 1988). This region of histone H4 seems to be involved in gene regulation since its (partial) deletion in yeast results in the derepression of the silent mating loci and in reduced expression of several genes including GAL1 and PHO5 (Kayne *et al.* 1988; Johnson *et al.* 1990; Park and Szostak 1990; Durrin *et al.* 1991).

### *The organization of histones and DNA in chromatin*

Woodcock (1973) and Olins and Olins (1974) showed first that chromatin can be visualized in the electron microscope as a string of bead-like particles, representing the first level, or nucleosomal, organization of DNA in chromatin. The core of nucleosomes, the histone octamere, consists of two molecules of each of the histones H2A, H2B, H3 and H4. A stretch of 146 bp of a DNA-double helix is wrapped in approximately 1.8 left-handed turns around the nucleosome core (Richmond *et al.* 1984). The central and C-terminal regions of the histones (with the exception of the C-terminal region of H2A) are involved in the structuring of the nucleosome core (reviewed by Wu *et al.* 1986; McGhee and Felsenfeld 1980).

A nucleosome core particle together with the "linker" DNA, the stretch of DNA between two core particles, is called the nucleosome. The length of the linker DNA varies (from 20-100 bp) between species (Compton *et al.* 1976; Morris 1976a; Noll 1976; Lohr *et al.* 1977), between different tissues (Compton *et al.* 1976; Morris 1976b) and in *Stylonychia* even between the macro- and micronuclei of a single cell (Lipps and Morris 1977).

The second step in the packaging of DNA in the chromatin is the folding of the 10 nm fiber, formed by the nucleosomes and the linker DNA, into a 30 nm fiber. This is achieved by an interaction of H1 with both the nucleosome core and the linker DNA. Besides histone H1, the N-terminal regions of the core histones are also suggested to be involved in the folding of the 10 nm fibril into higher order structures. The precise structure of the 30 nm fiber is, however, still a point of discussion. Several models have been proposed. According to the current view the solenoid model is the best explanation of the packaging of DNA at the level above nucleosomal organization (reviewed by Finch and Klug 1976; Eissenberg *et al.* 1985; Felsenfeld and McGhee 1986; Williams *et al.* 1986). According to this model the 30 nm fiber forms a single helix.

For packaging the total DNA of a eukaryotic cell (2 m for the human genome) into the nucleus, further folding of the 30 nm fiber is essential (Laskey 1986). A variety of models has been proposed for this higher order organization of chromosomes. The models differ in the mode how the 30 nm chromatin fiber is folded. However, the principle of the organization of chromatin in discrete and topologically independent domains is a generally accepted principle in most of them. Such domains are thought to be not only of organizational relevance but also of functional importance (reviewed by Eissenberg *et al.* 1985; Gasser and Laemmli 1987; Newport and Forbes 1987; see also Belmont *et al.* 1989). At present, attention is focussed on a scaffold-anchored loop model. The 30 nm fiber is supposed to form loops or domains of 30 - 100 kb of DNA, which are attached with their basis at a proteinaceous framework. Components of this framework are the peripheral lamina of the nucleus and an interior part, the nuclear matrix or nuclear scaffold. Also in metaphase chromosomes a scaffolding is seen (reviewed by Gasser and Laemmli 1987; Newport and Forbes 1987). Topoisomerase II is identified as one of the major proteins of the nuclear scaffold or nuclear matrix (Berrios *et al.* 1985; Earnshaw *et al.* 1985; Gasser *et al.* 1986). Furthermore histone H1 has been shown to bind selectively to scaffold attachment regions or matrix attachment regions (SARs/MARs, Izaurralde *et al.* 1989) and recently a chicken protein has been isolated which binds selectively to MARs from *Drosophila*, mouse and man (Kries *et al.* 1991). SARs/MARs are identified in a number of eukaryotes (e.g. *Drosophila*, chicken and man). SAR sequences in *Drosophila* have been localized near control elements of several genes (Gasser and Laemmli 1986) and have been characterized as AT-rich sequences (for review see Gasser and Laemmli 1987; Phi-Van and Strätling 1990). The SARs/MARs are presumed to form the basis of each of the chromatin loops. Recently it has been suggested that SARs/MARs determine the long-range conformation of chromatin domains via H1 assembly (Izaurralde *et al.* 1989).

The scaffold loop model is seriously criticised. An important argument is that the scaffold might arise artifactually by aggregation of proteins during the isolation procedure (see Eissenberg *et al.* 1985). Furthermore, the binding studies carried out with SAR/MAR sequences are *in vitro* studies which makes the significance of these sequences as *in vivo* origins of loop formation doubtful. On the other hand, there is little doubt on the existence of boundaries between functional domains within the chromatin which have been shown in the case of the silent mating type cassettes in yeast where they are represented by E and I sites (Abraham *et al.* 1984; Feldman *et al.* 1984). In addition, Kellum and Schedl (1991) showed with an *in vivo* assay the existence of functional domain boundaries in the 87A7 heat shock locus of *Drosophila*.

Whether there is a further higher order organization above the proposed scaffold loop structure is unclear. It has been suggested to exist in condensed chromosomes (Belmont *et al.* 1989; Mullinger and Johnson 1980; Rattner and Lin 1985; Tamigudin and Takayama 1986).

### *Histone variability and chromatin structure and function*

It is well established that the structure of chromatin in the cell nucleus in general reflects the function of the genetic material. Transcriptionally competent

or active chromatin, for example, has a more open structure. This is reflected in its increased DNase-I sensitivity whereas transcriptionally inactive chromatin (e.g. heterochromatin) and segregating chromosomes during cell-division form highly compact structures (for review see Eissenberg *et al.* 1985). However, how are chromatin structure and chromatin function correlated in detail? The primary interest concerns the question how chromatin structure can mediate gene expression. It must consequently been asked how the differences in chromatin structure are achieved. Since histones are the primary DNA packaging proteins, it is an acceptable idea that histones are involved in modifying chromatin structure. Stedman and Stedman (1950) were the first to propose that one of the functions of histones is to act as gene repressors. Primarily, the idea that histones may be involved in differential gene regulation became less attractive when the high degree of conservation in the primary structure of histones in the course of the evolution was discovered. However, with the development of improved separation techniques for histones, a considerable degree of variation within the subclasses of histones emerged. Gradually, the evidence for core histones and H1 as an integral component of regulator mechanisms of transcription increased (recently reviewed by Grunstein 1990a,b). This is, for example, illustrated by the displacement of nucleosomes in regulatory sequences upon hormone action (Bresnick *et al.* 1990) and by the prevention of initiation of transcription by the presence of nucleosomes on promoter sequences (see Grunstein 1990a and references therein). For H1 a difference in its interaction with the chromatin has been shown in active versus inactive chromatin (for review see Grunstein 1990a and references therein; see also Weintraub 1985; Shimamura *et al.* 1989). Furthermore, the individual core histones may have unique functions in gene regulation as it has been shown for histone H4 in yeast (see discussion on page 11).

It is easy to imagine that histone H1 variability or changes in the N-terminal regions of the core histones, which are the main sources of variability, influence the higher order folding of the 10 nm fibril. However, it has not been resolved yet how this histone variability can influence chromatin structure and function.

*Histone variability: sequence variants.* One type of histone variability is that of variation in the primary sequence. Sequence variants are generally found for both the linker histone and the core histones with the exception of H4 (see however Gatewood *et al.* 1990). The nonuniform distribution of histone variants within species is seen for example during embryonic development, among cell-types, or even within a nucleus (reviewed in Stein *et al.* 1984).

In sea urchins three different sets of histones ar synthesized during embryonic development. Cleavage stage variants substitute the histones of the oocyte and sperm nucleus. The replacement of these cleavage stage variants by the early histones starts already before the blastula stage. Subsequently, during the blastula stage substitution by the "late" histone variants begins (reviewed by Maxson *et al.* 1983).

Sea urchin species are the only species where subsequent transitions of histones during early development are found. The biological function of these histone replacements is still poorly understood. They might mainly be related to cellular differentiation.

For a number of histone sequence variants, however, their occurrence is clearly correlated to biological phenomena. Histone H5, a member of the family of linker histones, is an extreme example for tissue-specific variants. It is exclusively located in nucleated erythrocytes where it replaces most of the histone H1 (Neelin 1968). H5 deposition during the maturation of erythroid cells has been correlated to transcriptional inactivation, condensation of the chromatin and suppression of replication. *In vitro* studies showed that H5-chromatin has a higher compaction and stability compared to H1-chromatin, which is considered to be mainly due to the higher arginine content of the C-terminal segment of H5 (Kumar and Walker 1980; Thomas and Rees 1983). Furthermore, a change in nucleosome positioning due to the altered nucleosome repeat length correlated to H5 deposition, might be involved in transcriptional inactivation. The interaction of regulatory sequences of genes with transcription factors might be impaired by the altered nucleosome postions (Grunstein 1990a). Also the globular region of H5 probably is involved in the altered chromatin structure in comparison to H1-chromatin, since the strong conservation of the globular region in H1 variants is not seen between H1 and H5 histone variants.

$H1^{o}$, a third member of the linker histone family, accumulates in tissues that are terminally differentiated (Gjerset *et al.* 1982; Lennox and Cohen 1984). As is H5, $H1^{o}$ is associated with gene repression (Roche *et al.* 1985). This common "function" is reflected in the structure of H5 and $H1^{o}$. The sequence similarity between H5 and $H1^{o}$ is higher than between both $H1^{o}$ or H5 and H1 (see Wells and McBride 1989). The lower arginine content in the C-terminal domain of $H1^{o}$ in comparison with H5 suggests a weaker interaction of $H1^{o}$ with chromatin (Doenecke and Tönjes 1986). Sequence variation in core histones is mainly present in the N-terminal regions (Wells 1986; Wells and McBride 1989). These regions are also the main targets of modifications of the core histones. The N-terminal tails of core histones, as already mentioned, are probably involved in the stabilization of the nucleosomes. Nucleosome unfolding or displacement has to occur *in vivo* in order to allow initiation of transcription. For H2A the variants H2A.X and H2AF/Z not only differ with respect to the N-terminal region. These variants are found highly enriched in transcriptionally active chromatin. A correlation of this function with their structure has not yet been made.

Analysis of the expression of the sequence variants has led to the conclusion that there are at least two separate classes of somatic histone genes, namely those whose expression is correlated with DNA synthesis, and those which are expressed even in the absence of DNA replication. The former class is also called replication(-dependent) variant class, the latter basic or replacement variant class (Wu and Bonner 1981; Schümperli 1986).

Cell-cycle-dependent expression of genes coding for the replication variants is mediated by a variety of transcriptional and posttranscriptional regulatory mechanisms. In the replication-dependent transcription of these genes several promoter elements are involved interacting with *trans*-acting regulatory factors (e.g. Sive *et al.* 1986; Fletcher *et al.* 1987; Dalton and Wells, 1988; Tung *et al.* 1990). In addition to transcription the 3'-end formation and 3'-end structure of these messengers is contributing to the cell-cycle regulated expression. The 3'-ends of mature mRNAs are formed by endonucleolytic cleavage of the primary transcripts (reviewed by Birnstiel *et al.* 1985). Two sequences of this end of the gene, a highly conserved palindrome and a less conserved purine-rich sequence

immediately downstream, are involved in this process. In addition three factors acting in *trans* have been identified: the U7 small nuclear ribonucleoprotein particle (U7 SnRNP), a heat labile factor and a hairpin binding factor (Hoffmann and Birnstiel 1990 and references therein). In most organisms the genes coding for replication variants occur clustered in the genome.

The less prevalent replacement variants substitute for the major replication variants mainly in differentiating and quiescent cell-types. Genes coding for replacement variants are distinguished from the replication-dependent genes by several structural features. They are not clustered but appear dispersed in the genome, mostly as single copy genes. They lack the promoter elements responsible for the increase of transcription in S-phase. Their transcripts are polyadenylated and the 3'-end elements described above for the replication variants, are missing. Moreover, introns are shown to be present in the replacement genes (Engel *et al.* 1982; Krieg *et al.* 1982; Harvey *et al.* 1983; Brush *et al.* 1985; van Daal *et al.* 1990). Tissue-specific histone variants, like histone H5 present in nucleated erythrocytes, are either mentioned as a separate class (Schümperli 1986) or together with the replacement variants (Challoner *et al.* 1989). Although tissue-specific variants are expressed indepent to the cell-cycle, part of the genes have in their 5'- and 3'- regions sequence elements characteristic for the replication-dependent genes (Busslinger and Barberis 1985; Barberis *et al.* 1987; Hwang and Chae 1989). For the testis-specific H2B-1 of sea urchin it is shown that these 5' (promoter) elements are involved in the tissue specific expression of the genes (Barberis *et al.* 1987).

The recent discovery that two independently regulated mRNAs, namely a polyadenylated messenger and a messenger ending in the typical 3'-hairpin structure, can be transcribed from the same "cell-cycle dependent" gene, showed that the separation of replication- and replacement variant genes is not that strict (Challoner *et al.*, 1989; Cheng *et al.*, 1989; Kirsch *et al.*, 1989). A fine mechanism of regulation of the expression of the different histone genes involved in the differential organization of chromatin within a cell, is more and more elucidated.

*Histone variability: posttranslational modifications.* The second type of variability of histones, is mediated by their posttranslational modification(s). The 5 main types of partly reversible modifications of histones are acetylation, phosphorylation, methylation, ADP-ribosylation and ubiquitination (reviewed by Isenberg 1979; McGhee and Felsenfeld 1980; Wu *et al.* 1986). Most research on posttranslational modifications of histones has been directed towards acetylation and phosphorylation.

In the case of acetylation two modes can be distinguished. The first kind, an amino-terminal acetylation is usually found for H2A, H4 and H1 and it is irreversible. The second kind is the reversible side chain acetylation. This type of acetylation wich mainly occurs in the N-terminal tails of the core histones, has been correlated with the onset of a variety of nuclear processes, including transcription, replication, chromatin assembly and the replacement of histones by protamines or protamine-like proteins (for review see Wu *et al.* 1986; Loidl 1988; Csordas 1990; Pfeffer and Vivaldi 1991; see also Thorne *et al.* 1990). The dominant mutation *Su-var(2)1*$^{01}$ in *Drosophila* shows suppression of position effect variegation. The hyperacetylation of histone H4 which is found in

these mutants (larvae) suggests that deactylation of H4 is necessary for chromatin condensation (Szabad *et al.* 1988).

Histone **phosphorylation** is mainly studied in its relation to mitosis (Shibata *et al.* 1990 and references therein; for review see Wu *et al.* 1986). A coupling between the phosphorylation of particular H1 sites and subsequent chromosome condensation during G2 is seen. Also histone H3 molecules are in a phosphorylated state late during G2 and during metaphase.

Histone phosphorylation has been found for the sperm-specific histones H1 and H2B of sea urchins at the time of their deposition in the sperm head and immediately after fertilization when decondensation of the chromatin and replacement of the testis-specific by somatic histones occur (reviewed by Poccia 1986, 1987). H1 phosphorylation has also been proposed to be involved in gene regulation (Susuki *et al.* 1990). An example of this might be seen in the phosphorylation of a specific subtype of H1 which occurs during the initial phase of differentiation of mouse neuroblastoma cells (Ajiro *et al.* 1990)

**Ubiquitination**, a covalent linkage to the protein ubiquitin, is seen for histone H2A and H2B and their variant forms. There is evidence that ubiquintinated H2A and H2B are associated with transcriptionally active chromatin (Levinger and Varshavsky 1982; Barsoum and Varshavsky 1985; Nickel *et al.* 1989; Davie *et al.* 1991). Only for H2B the level of ubiquitination seemed to be coupled to ongoing transcription. Ubiquitination of H2A is also seen in transcriptionally competent chromatin (Davie and Murphy 1990).

**Methylation** of histones has been proposed to play a role in chromatin condensation or mitosis (Honda *et al.* 1975). Furthermore transcriptionally active or competent chromatin is enriched in newly methylated histones H3 and H4 (Hendzel and Davie 1989, 1991 and references therein).

**ADP-ribosylation** of histones seems to play a role in processes involving DNA strand breaks such as repair, replication or recombination or in processes accompanied by changes in the superhelical turns of chromatin loops (summarized by Boulikas 1990).

Although the above mentioned examples show the coincidence of the modification of histones with biological functions of chromatin, a causal link between the modifications and regulatory processes in chromatin has still not been proven experimentally, in particular the physiological consequences of histone modifications are still not understood (for review see Csordas 1990).

The variability of histones, in addition to a variable interaction with DNA and with each other, is likely to result in a variable interaction of histones with nonhistone proteins such as the high mobility group proteins. Recently a *Drosophila* gene has been isolated whose copy number influences the extent of position effect variegation (Reuter *et al.* 1990). The widely spaced zinc-finger structure of this protein may function in the packaging of heterochromatin through interaction with other proteins, probably histones. Histone variants may differ in their interaction with for example this protein.

### *Histones and histone genes in Drosophila*

Histone genes of *Drosophila melanogaster* were among the first histone genes to be cloned and characterized (Karp and Hogness 1976). However, further

research on histone genes and also on histone proteins in *Drosophila* was very limited in comparison to that in sea urchins, *Xenopus* or mammals.

Histone genes of *D. melanogaster*, the main species for research on histone genes in *Drosophila*, are organized in the genome as tandem repeats with approximately 110 copies. Each repeat unit contains one copy of the genes coding for the histones H1, H3, H4, H2A and H2B. In contrast to some other organisms, the transcription of the individual histone genes occurs not from one DNA strand in *Drosophila*. H1, H2A and H3 are transcribed from the same DNA strand, while H2B and H4 are transcribed from the opposite strand and display consequently an opposite direction of transcription.

Two main types of repeat units with a length of 4.8 kb and 5.0 kb are present. These differ in the length of the spacer region between the H1 and H3 genes by an insertion of 240 bp long DNA sequence, which appears to be derived from a tRNA gene (Matsuo and Yamazaki 1989). Both types of repeat units are mainly organized in homogeneous blocks (Lifton *et al.* 1977; Strausbaugh and Weinberg 1979; Saigo *et al.* 1980). Strains of *D. melanogaster* differ in their relative amounts of the two repeat types. The 5.0 kb repeats are always present in an equal or larger number than the 4.8 kb repeat (Strausbaugh and Weinberg 1982).

The tandem organization of histone genes in *D. melanogaster* is comparable to that of the genes coding for the early histone variants in sea urchin (Cohn *et al.*, 1976). It is estimated that the number of histone genes present in the *D. melanogaster* genome exceeds the cellular requirements about tenfold (Anderson and Lengyel 1984). This raises the question whether some portion of the histone genes is differentially expressed, as it has been found in sea urchins. Evidence for a possible subdivision of the histone cluster in the 2nd chromosome of *D. melanogaster* was derived from the banded patterns of grains (region 39 DE) after *in situ* hybridizations with $^{3}$H-labelled histone mRNAs as a probe (Pardue *et al.* 1977). The organization of the histone genes into 14 subsets, separated by non-histone DNA (Saigo *et al.* 1980) was also suggestive with respect to a functional subdivision of the histone cluster. However, despite of extensive research Anderson and Lengyel (1979, 1980, 1981, 1984) found no additional indications for changes in the histone messenger population during embryogenesis - in contrast to the changes found in sea urchins. Also the maternal histone messengers in the egg do not detectably differ from newly synthesized histone mRNA's in the embryo (Anderson and Lengyel 1984). It appears, thus, that the same type of histones is transcribed in *Drosophila* embryos and adults. Only genes coding for histone variants and transcribed at very low levels or messengers with a low degree of sequence similarity to the histones encoded in the main cluster may have escaped detection. The results of the restriction analysis of histone coding DNA and its organization in nucleosomes during early development and in culture cells (Samal *et al.* 1981), agree with this conclusion on the absence of differing subsets of histone genes.

Genetic analysis so far is insufficient to decide whether different functional units may be present within the histone repeat cluster (Siegel 1981).

Although histone messengers are not detectable, a H2A variant, called D2, represents approximately 10% of the total H2A histone in both *D. melanogaster* embryos and adults (heads) (Alfageme 1974; Palmer *et al.* 1980; Donahue *et al.* 1986). In *D. virilis* a homologous protein seems to be present (Palmer *et al.*

1980). Analysis of a *D. melanogaster* embryonic cDNA clone and of the genomic sequences coding for H2AvD, most likely representing D2, revealed that this histone variant fits into the scheme of replacement variants (van Daal *et al.* 1988, 1990). The gene contains introns, transcripts are polyadenylated and the 3′-end palindromic sequence is absent. According to the amino acid sequence deduced from its cDNA, the H2AvD variant belongs to the evolutionarily conserved H2A.F/Z type of H2A variants which has been discovered in *Tetrahymena*, sea urchins, chicken and mammals. Also the conservation of the position of introns in the corresponding genes is characteristic of this histone H2A variant class (van Daal *et al.* 1990). The H2AvD gene is located outside the histone gene cluster. Its messenger RNA has not been detected in the study of Anderson and Lengyel (1979, 1980, 1981, 1984) probably due to the low degree of sequence similarity between this mRNA and the sequence of the presumptive cell-cycle regulated H2A gene.

For H1 there are indications for the existence of sequence variants in *D. virilis* (Blumenfeld *et al.* 1978a,b). They have been suggested on basis of separation in protein gel electrophoresis, but no amino acid or nucleic acid sequences are available yet to confirm this.

There might also be genes coding for variants of the histones H2B and H3 in *D. melanogaster* since Childs *et al.* (1981) described the presence of sequences in the genome of *D. melanogaster* outside the histone gene cluster, with similarity to the H2B or H3 genes.

The summary of the data on histones in *Drosophila* shows no evidence for the existence of stage- or tissue-specific histone variants. It is possible, that heterogeneity of histones during embryogenesis and in different tissues is based on posttranslational modifications and on differences in the amount of histone H1 relative to the core histones (Oliver and Chalkley 1972; Blumenfeld *et al.* 1978 a,b; Holmgren *et al.* 1985 Harisanova and Ralchev 1986 a,b). Alternatively, variants have not yet been detected because of their low abundance or because of the low sequence similarity of their messengers to the DNA used as probe.

### *Histones and spermatogenesis*

The preceeding sections of this chapter gave an overview on histone variability, their function in chromatin organization and their correlations to biological processes. The following discussion will be focussed on spermatogenesis. Fundamental changes in histone complement and chromatin organization have been observed in this developmental process for many organisms.

During spermatogenesis, germ cells differentiate into male gametes. The basic pathway of this differentiation can be divided into three intervals: (1) the premeiotic phase which includes the mitotic stem cell divisions, proliferation and commitment to differentiation; (2) meiosis where reduction to haploidy occurs; and (3) spermiogenesis, the morphogenetic process during which a spermatid acquires the complex structure of a sperm cell. Chromatin in the developing sperm cells has to meet the requirements of differential gene expression during the differentiation process, it has to pass through a reduction division and finally it has to acquire the compact structure as found in the mature sperm head. This compaction is achieved by drastic changes in chromatin organization. The somatic type of histones responsible for the primary

compaction of the DNA in somatic cells and early germ line cells by forming nucleosomes are replaced either by testis-specific or by testis-enriched histones, more basic than the somatic histones, and/or by highly basic small proteins such as protamines. There are only a few exceptions known as for example the goldfish *Carassius auratus* which retains the somatic type of histones in sperm nuclei (Munoz-Guerra *et al.* 1982).

*Classification of basic sperm proteins*

The basic nuclear proteins of mature sperm display a very high degree of diversity in different organisms. Bloch (1969) introduced a classification of basic nuclear sperm protein complements mainly based on cytochemical data. He distinguished:
(1) the salmon-type, with sperm containing protamines rich in arginines (e.g. trout, salmon, rooster),
(2) the mouse- and grasshopper-type with protamines rich in arginines and cysteines (e.g. eutherian mammals, grasshopper),
(3) the *Mytilus*-type with sperm proteins with an arginine- and- lysine content intermediate to that of histones and protamines (e.g. echinoderms, molluscs, *Xenopus laevis*),
(4) the *Rana*-type with histones as the basic sperm proteins and
(5) the crab-type with no detectable basic nuclear proteins (e.g. the crab *Libinia emarginata*). Biochemical analyses (reviewed by Poccia 1986; see also Risley 1989) showed heterogeneity in the sperm protein classes and also relationships between them. A strict separation of the classes 3 and 4 is often not possible. They may better be regarded as one heterogeneous group (Poccia 1986). Such a classification appears more adequate as, for example, the sperm of the winter flounder, *Pseudopleuronectes americanus*, contains a somatic type of histones together with a family of high molecular weight proteins (Kennedy and Davies 1980), or the marine bivalves, *Swiftopectin swifti* and *Glyssymeris yessoensis*, possess a very heterogeneous set of sperm proteins (Zalenskaya *et al.* 1985). The sperm of these species contains low molecular weight basic proteins in combination with sperm-specific arginine-rich H1 and somatic histones.

Also in the classes 1 and 2 the packaging of DNA by protamines is not as homogeneous as it initially was thought. The sperm of trout (Avramova *et al.* 1983) and ram (Uschewa *et al.* 1982), for example, contains, in addition to protamines, small amounts of tightly bound core histones. In human sperm, about 15% of the histone complement of a haploid genome is present, besides a mixture of protamines and intermediate basic proteins (Tanphaichitr *et al.* 1978; Gusse *et al.* 1986). Alternative classifications have been proposed by Subirana (1983) and Kasinsky and coworkers (1985). All these classifications must be considered as purely descriptive, since clear evolutionary trends in sperm proteins of different species are not obvious (see Poccia 1986 and references therein).

Poccia (1986) gave an extensive compilation of the different sperm proteins and their characteristics. The structure of protamines has more recently been reviewed by Hecht (1989 a,b). I will subsequently confine myself to histones found during spermatogenesis and in mature sperm.

*Chromatin structure in sperm*

The variation in sperm nuclear proteins, is reflected in the variation of the chromatin structure of the sperm nuclei (reviewed by Poccia 1986; Risley 1989; Hecht 1989a). The characteristic nucleosomal configuration of DNA is only observed when all four core histones are present (Subirama 1983; Poccia 1986). In this case the overall nucleosomal organization in sperm nuclei differs from that in somatic cells by an increased nucleosome DNA repeat length (e.g. sea urchin, Spadafora *et al.* 1976; Keichline *et al.* 1979, and winter flounder, Kennedy and Davies 1982). Puigdomenech and coworkers (1987) showed that the increase occurs in the linker DNA. This increased spacing of nucleosomes is correlated with the presence of large testis-specific H1 histones. Only in winter flounder sperm chromatin there is no testis-specific histone H1. In this organism the high molecular weight proteins might be responsible for the increased spacing of nucleosomes. Exceptions are the goldfish and carp, where only the somatic type of histones are present in the sperm nuclei (Munoz-Guerra *et al.* 1982; 1987; Kadura *et al.* 1983)

The higher order organization of the sperm DNA organized in nucleosomes is reflected by fibers covered with superbeads of 21 - 48 nm in diameter, with their sizes depending on the species (for review see Risley, 1989). The diameter in sea urchins greatly exceeds that of other species. This has been attributed to the greater ability of sperm specific H1 and H2B to form internucleosomal crosslinks.

The structural organization of DNA in sperm with protamines or with proteins of a character intermediate between histones and protamins is less clear (for review see Hecht 1989b; Risley 1989). The high compaction of such DNA-protein complexes makes *in situ* analysis difficult. Moreover, artefacts are easily introduced by the harsh methods necessary to decondense the sperm nuclei. Both X-ray diffraction studies and nuclease digestion have revealed that at least the major part of the chromatin looses its nucleosomal organization when the histones are replaced by more basic proteins. Nucleoprotamine fibers with arginine-rich protamines (class 1) must form a very regular structure since it appears almost crystalline from X-ray diffraction analysis. The fibers seem to be condensed in parallel bundles forming a hexagonal array.

Sperm chromatin containing proteins from the second, third and fourth class are less regularly organized. The chromatin in such cases may have a non-uniform structure being organized in different types of DNA-protein complexes.

In both human sperm and *Mytilus* sperm (Zalensky and Avramova 1984) nucleosomal histone complexes and nucleoprotamine complexes seem to be present simultaneously. This might also hold true for trout and ram sperm where small amounts of tightly bound core histones have been found. For human sperm it has been demonstrated that paricular sequences seem to be preferentially attributed to either the nucleohistone or to the nucleoprotamine fraction (Gatewood *et al.* 1987, see below).

The higher order organization of nucleoprotamine filaments replacing the 10 nm nucleosomal filaments is less well defined. Despite of a high degree of diversity in higher order fibers, three major patterns of condensation have been identifled: (I) a the fibrous, (II) a lamellar and (III) a granular structure

(for review and discussion see Hecht 1989b; Risley 1989). Sperm nucleoprotamine filaments may be further organized in structural domains, nonhistone proteins being involved in their organization. However, detailed insight into the structural conformation of sperm chromatin is still very scarce (see Risley 1989 for an extensive discussion).

*Function of histone transition during spermatogenesis*

Changes in the histone composition during spermatogenesis seem to occur in most animal species. Compilations of theoretically possible functions of this process have repeatedly been provided (reviewed by Bloch 1969; Poccia 1986; Hecht 1989a; Risley 1989), but clear evidence for its actual importance has hardly been given.

The following functions of histone transition have been proposed:

(1) Basic sperm proteins are involved in the shaping of the nucleus "designing" a sperm nucleus with a hydrodynamically efficient shape. However, there are indications that the shape of the sperm head is hydrodynamically insufficient and that the shaping of the nucleus occurs independent of the DNA condensation. Furthermore, a correspondence between nucleoprotein type and headshape is not obvious.

(2) The basic sperm proteins are involved in the transcriptional inactivation of the chromatin. However, transcriptional inactivation, where studied, occurs before the final deposition of these basic proteins (reviewed by Poccia 1986). A function in inhibition of transcription by the testis-specific histones H1 and H2B in sea urchin is recently discussed again by Suzuki (1989). Since the dephosphorylation of the sequence motifs suggested to be responsible for this inactivation, occurs only late in spermiogenesis (Hill *et al.* 1990, and references therein) transcriptional inactivation is also in this case not a likely primary function for histone transition.

(3) The basic sperm proteins have a protective function during storage of sperm or during internal or external fertilization. Although some authors report on higher stability of sperm chromatin with respect to DNase sensitivity, thermal denaturation, ionic unwinding or sonification, there is no straightforward correlation of the type of the sperm nucleoprotein with the length of sperm storage or with the type and environment of fertilization. Furthermore, the protection of sperm and late spermatid DNA against chemical mutagens and DNA damage by X-rays seems to be rather weak.

(4) Basic sperm proteins or basic proteins occurring during spermatogenesis might be involved in genomic imprinting. The term imprinting refers to a process in male or female gametogenesis resulting in a differential function of specific genes or chromosomal regions in the offsprings, depending on their paternal or maternal origin. Methylation of CpG sequences is supposed to have a function in imprinting but it is quite possible that this methylation is not the primary event but a consequence of imprinting. On the other hand, the sequence-specific packaging of the human sperm DNA in either nucleohistone or nucleoprotamine complexes might indicate a programming of these sequences for very early expression during embryogenesis (Gatewood *et al.* 1987, 1990). However it is an open question, whether histones or more in general, chromatin packaging during spermatogenesis, have a function in (the conservation of) imprinting.

(5) The mode of packing DNA in sperm chromatin might have to meet special requirements of the processes in the male pronucleus after fertilization. The regionalized nucleohistone complexes in human sperm might, for example, be sites of initiation of decondensation of the chromatin in the male pronucleus (Gatewood *et al.* 1987).

It is puzzling that, although much on the variability and at least part of the structure and its physiological consequences of sperm chromatin are known, no obvious correlations can be made with these properties and biological function or with the evolutionary position of different organisms. It has been suggested that in contrast to the limited variability of histones in somatic cells, a large variability is tolerated in sperm cells because the evolutionary constraints imposed by the normal chromatin functions (e.g. replication, transcription) are lacking (for discussion see Kasinsky *et al.* 1978; Poccia 1986). It is, however, difficult to imagine that this would lead to the establishment of additional functional genes with tissue-specific expression and to large-scale rearrangements of the chromatin.

It is evident, that more detailed knowledge on sperm chromatin organization and the biophysical properties of nucleoprotein complexes are necessary to elucidate the correlation between structure and function of sperm chromatin. Knowledge on the mode of revision of the sperm chromatin into a functional state after fertilization may in addition give insight in the mechanisms of imprinting.

In the following section I will confine myself to the histones present during spermatogenesis and in mature sperm.

### *The process of histone transition*

The pattern of changes in the chromosomal protein complement during spermatogenesis in different organisms is as diverse as the nature of sperm proteins (for review see Poccia 1986; Hecht 1989a,b). In some species a "one step" transition occurs where part of the somatic histones are directly replaced by sperm-specific histones (e.g. sea urchin). In the winter flounder, on the other hand no replacement of somatic histones takes place, but additional basic proteins are added at a certain stage of spermatogenesis. The most complex array of transition events has been described for eutherian mammals,which include mouse, rat and man (for review see Hecht, 1989a,b).

Since the most detailed analyses of the different aspects of histone transition (e.g. time of transition, protein composition, chromosome and gene structure as well as regulation) have been done for sea urchins, rat, and mouse I will confine myself in the following part to these species. Especially in sea urchins recent data are informative with respect to integrating structural features of the sperm- specific proteins with special properties of sperm chromatin. They also show how the interactions of these proteins with the DNA are modulated in the course of spermatogenesis.

*Sea Urchin.* In chromatin of sea urchin sperm the somatic H1 and H2B are replaced by the sperm-specific histones SpH1 and SpH2B. Two different histones of the SpH2B-type were found in different sea urchins, differing in their relative amounts between different species (Busslinger and Barberis 1985, Lieber *et al.* 1986). *Parechinus angulosus* contains even three sperm-specific H2B

histones (Strickland *et al.* 1977a,b, 1978). Although two different testis-specific H1 transcripts were reported for *Strongylocentrotus purpuratus* (Lieber *et al.* 1986), only a single sperm H1 subtype has been described in detail so far The remaining core histones H2A, H3 and H4 in sea urchin sperm are in general of the somatic type (Easton and Chalkley 1972; Busslinger and Barberis 1985). However, in the sperm of *Psammechinus miliaris* and *Parechinus angulosus* an H2A variant (H2A-3) is found which is also expressed during the late embryonic development (Wouters *et al.* 1978; Strickland *et al.* 1980; Busslinger and Barberis, 1985). SpH1 and SpH2B are accumulated in spermatogonia and primary spermatocytes (Poccia *et al.* 1987; Poccia *et al.* 1989). Towards the end of spermiogenesis the chromatin becomes highly condensed but remains organized in nucleosomes. This organization is retained in mature sperm. The chromatin is in this case characterized by an increase in length of the linker DNA in sperm chromatin compared with somatic cells (Puigdomenech *et al.* 1987).

Sea urchin SpH1 and SpH2B proteins are the best-studied among the sperm-specific histones, especially with regard to functional aspects. The main differences between SpH1, SpH2B and their somatic counterparts are N-terminal extensions present in the sperm-specific histones. These extensions consist of short repeats, which can be considered as variants of the tetrapeptide sequences Ser-Pro-basic-basic or basic-basic-Ser-Pro, where the "basic" constituents are either lysine or arginine. In SpH1 the same amino acid sequences also occur in several repeats at the carboxy-terminal end of the protein. These repeats are, however not in a tandem array, but interspersed (for compilation of these sequences see von Holt *et al.* 1984; Poccia 1987)).

During the initial stages of spermatogenesis SpH1 and SpH2B are phosphorylated. In the late stages of maturation of the spermatids a dephosphorylation takes place. Sites of phosphorylation are the serine residues in the N-terminal extensions. SpH1 is also phosphorylated in its C-terminal region (Poccia *et al.* 1987; Hill *et al.* 1990). The chromatin condensation in late spermatids precedes the dephosphorylation of SpH1 and SpH2B. This indicates that not the tetrapeptide repeats are responsible for this condensation. This condensation is probably achieved by the C-terminal domain of SpH1, which is highly basic and for a large part unphosphorylated (Hill *et al.* 1990). The C-terminal domain of H1 is known to bind to and to condense the linker DNA between the nucleosomes (Allan *et al.* 1986). Hill and coworkers carried out structural analyses on the C-terminal regions of SpH1 and showed that both *in vivo* and *in vitro* the proline-free, most N-terminal region of the C-terminal domain has an α-helix structure. The structure of the remaining part of the C-terminal region of SpH1 is still unknown (for discussion see Clark *et al.* 1988; Hill *et al.* 1989). The time of dephosphorylation of SpH1 and SpH2B suggests the N-terminal domains of the sperm-specific histones to have their role in the stabilization of the highly condensed structure of sperm chromatin by a strong electrostatic binding to the extended linker region of the DNA (Green and Poccia 1988; see also Poccia 1987). Linkage was proposed within the chromatin fibers or between chromatin fibers (von Holt *et al.* 1984; Green and Poccia 1985, 1988). The stabilization of the condensed sperm chromatin is reflected in a higher thermal stability and a higher resistance to micrococcal nuclease digestion (Green and Poccia 1988). No comparisons with the somatic H1 were made.

A special type of β-turn has been proposed as structure of the Ser-Pro-basic-basic motifs. Ser-Pro-basic-basic peptides were shown to bind to the minor groove of the DNA double helix, with a preference for AT-rich sequences. Most likely the Ser-Pro-basic-basic motifs bind to the linker DNA (Suzuki 1988, 1989; Churchill and Suzuki 1989). The stabilization of the superhelical coiling of the nucleosomal chain as a consequence of neutralization of the negative charges on the inside the DNA superhelix has been implied (Churchill and Suzuki 1989). Whether this holds true *in vivo* remains to be proven. A similar mode of binding and neutralization of negative charges has been suggested for protamines, integration host factor and HU proteins (Mirzabekov and Rich 1979; Balhorn 1982; Churchill and Suzuki 1989; White *et al.* 1989; Yang and Nash 1989). By phosphorylation of the serine of the tetrapeptide motifs the ability of the charge neutralization would be diminished and as a consequence DNA-binding decreased. A decreased binding affinity to DNA, caused by phosphorylation, may result in the function of sperm-specific histones as typical histones throughout spermatogenesis (Poccia *et al.* 1987).

*Rat/mouse.* The second group of animals, where a more detailed knowledge on histone transition during spermatogenesis exists, are the eutherian mammals, in particular mouse and rat. The changes in the basic nuclear protein complement during spermatogenesis are much more complex in eutherian mammals than in sea urchins and the changes occur in a multistep process.

Variants for all histones except H4 appear during the mitotic and meiotic phases of spermatogenesis in rat and mouse. In rat the testis-enriched histones H1a and H2A.X already accumulate during the spermatogonial stage (Meistrich *et al.* 1985). With some minor deviations, probably due to methodical differences, this is consistent with data reported for mouse spermatogenesis (Bhatnagar 1985; Rasheed *et al.* 1989). In both species, the testis-specific H3 (TH3) which has not been found in other mammals, is present in mouse from the spermatogonial stage, in rat from the primary spermatocyte stage. The relative amounts of these variants decrease from or after the pachytene primary spermatocyte stage (Zweidler 1984; Meistrich *et al.* 1985).

The testis-specific variants, H1t, TH2A and TH2B appear somewhat later in rat spermatogenesis than the above mentioned variants. H1t does not accumulate until the pachytene of the primary spermatocyte stage. This holds also true for mouse spermatogenesis (Bucci *et al.* 1982; Bhatnagar *et al.* 1985). TH2A and TH2B are clearly present in early primary spermatocytes, and it cannot be excluded that low levels of TH2A and TH2B are synthesized in spermatogonia (Meistrich *et al.* 1985). The TH2B variant seems generally to be present in mouse, whereas a testis-specific H2A variant appears to be limited to some species. Although long known, both variants have not been well characterized until now (Zweidler, 1984).

A second testis-specific H2B variant in mouse, the spermatid-specific H2B (ssH2B) has been described by Moss *et al.* (1989). The messengers are synthesized either late in meiosis or during the round spermatid stage. The presence of the ssH2B mRNA in polyribosomes strongly suggests that the ssH2B messengers are translated in round spermatids although the protein so far has not been identified in testis. Whether a histone homologous to ssH2B is present in rat or other mammals is not known.

As an intermediate step during spermiogenesis, histones are replaced by transition proteins (TPs). The TPs first occur in elongating spermatids and

their presence is closely correlated with the condensation of chromatin. With this transition the nucleosomal structure of the chromatin is lost. For some mammals the substitution of histones by protamines is not complete (e.g. human: Tanpaichitr *et al.* 1978; Gusse *et al.* 1986, mouse: O´Brien and Bellvé 1980), but the extent to which this occurs in other mammals is not known.

Transition proteins are small proteins (with a molecular weight less than 20,000 Da), and they are more basic than histones. Although they are similar in their amino acid composition, they are considerably different in their amino acid sequence as far as it can be derived from the limited sequence information available. The number of TP variants, found among species, differs. In rat not more than four different TPs are present As for all basic nuclear sperm proteins, the function of TPs is still unclear. Most likely they have an intermittent function in chromatin condensation (for discussion see Hecht 1989a), but also some importance for transcriptional inactivation has been considered (for review on transition proteins see Poccia 1986; Hecht 1989a).

Toward the end of spermiogenesis the protamines are synthesized. They finally replace the transition proteins, and persist in mature mammalian sperm. The protamines form a particular class of small highly basic proteins. In eutherians they are rich in arginine and cysteine. While most mammals are believed to contain only one protamine (e.g. rat, bull, ram), in mouse and human a second protamine fraction has been discovered (for details on structure and function of the protamines and the corresponding genes see Hecht 1989a,b). It cannot be excluded that additional protamines may generally be present.

The ideas about the function of different testis-specific or -enriched histone variants are mainly based on their appearance during specific stages of spermatogenesis. H1a, H2A.X and TH3, present already in spermatogonia in high amounts, might have a function in programming of spermatogonia for spermatogenesis, a role in the mitotic divisions or in the preparation of the chromosomes for meiosis (Meistrich *et al.* 1985). A correlation between the presence of H1a in the chromatin and the proliferative state of the cell has also been suggested by Lennox and Cohen (Lennox 1984; Lennox and Cohen 1988; see however Rasheed *et al.* 1989). Rasheed *et al.* (1989) suggested a role of H1a in selective repression of genes (*cf.* Wolffe and Brown 1988). Since there are no structural data available nor have experiments concerning its function been performed further conclusions cannot be drawn.

For the H2A.X variant the amino acid sequence of the human homologue is known (Mannironi *et al.* 1989). The H2A.X and the predominant human H2A.1 variant are almost identical in the first 120 residues, but unrelated beyond residue 120. The sequence similarity between the C-terminal region of H2A.X and of H2A of several lower eukaryotes led to the hypothesis that H2 A.X has a function related to the transcriptionally active state of chromatin (Mannironi *et al.* 1989). This is consistent with the occurrence of H2A.X in early spermatogenic stages with high transcriptional activity.

TH3 differs from somatic H3 in several positions of its C-terminal region (Zweidler 1984), the region involved in histone-histone interaction. This suggests an influence on nucleosome structure. The consequences for chromatin structure and function are unknown.

The amino acid sequence of rat H1t where arginine replaces lysine, suggests a stronger binding of the C-terminal domain to the linker DNA compa-

red to somatic H1 (Cole *et al.* 1986a and references therein). Although there exists generally a great tolerance for inter-species variation within this H1 class (Cole *et al.* 1986a,b), there is a characteristic pattern of divergence of H1t from the sequence of standard somatic H1 proteins. H1t of rat and boar have a shorter C-terminal region in common as emerged from a comparative study with boar H1t which is also found for histone H5 (Cole *et al.* 1984). In addition to the short C-terminal region, rat and boar H1t show much more divergence within the usually conserved central globular region; the region which is assumed to seal the two turns of DNA around the nucleosome (Allan *et al.* 1980).

The period of accumulation of H1t in rat spermatogenic cells, during mid to late meiotic prophase, induced Meistrich and coworkers (1985) to suggest a role of H1t in separation of the paired chomosomes, gene expression in spermatids or in the condensation of the spermatid nucleus and replacement of histones by transition proteins. An enrichment of H1t in repair domains of chromosomes suggest a role for H1t in the meiotic repair mechanisms (Markose and Rao 1989). The substitution of arginine for lysine in the C-terminal region of H1 implies a stronger binding to the linker DNA which fits to the proposed role of H1t in chromosome condensation or transcriptional inactivation. Cole *et al.* (1986b) described possible structural consequences of the differences in amino acid sequence between testis-specific and somatic H1. Extrapolations to functional consequences were, however not made.

The amino acid sequence of rat testis-specific H2B (=TH2B) and a somatic H2B were deduced from cloned cDNAs. They showed extensive difference in the N-terminal 40 amino acids while the C-terminal two third of the H2Bs are almost identical (Kim *et al.* 1987). The most significant difference is seen in the first 12 amino acids and in the presence of a cysteine in TH2B. The C-terminal two thirds of both H2Bs are almost identical. This region is most likely involved in the binding to DNA (for review see Isenberg 1979; McGhee and Felsenfeld 1980). The difference in structure seem to be reflected in a loosened nucleosome structure of pachytene spermatocytes in comparison to liver chromatin (Rao *et al.* 1983) and a higher DNase I sensitivity of the H2B binding site in nucleosomal DNA.

As for the other testis-specific or testis-enriched histones it remains to be determined whether the presence of TH2B and an altered nucleosome structure is necessary for biological processes such as chromosome pairing, genetic recombination or activation of testis-specific genes. A relaxed nucleosome structure might facilitate replacement of the histones late during spermiogenesis. The predicted amino acid sequence of the spermatid-specific H2B in mouse gives an indication for a function of the putative protein (Moss *et al.* 1989). The amino acid sequence is almost identical to a somatic H2B, but shows a C-terminal extension. The hydrophobicity of this extension is similar to that of the amino- terminal signal peptide sequence of secretory proteins. The signal peptide of ssH2B might direct the protein to the nuclear membrane as is known for other integral membrane proteins (Sabatini *et al.* 1982). The ssH2B, found at the nuclear periphery, might have a function in organizing the chromatin prior to its condensation. The process of chromatin condensation in the mouse spermatid nucleus initiates along the inner membrane of the nuclear envelope (Dooher and Bennett 1971). These starting points of condensation may be coincident with sites of chromosome attachment to the

nuclear membrane (*cf.* the location of large heterochromatin blocks at the nuclear envelope in mouse spermatids, liver cells and Sertoli cells: Rae and Franke 1972). It has to be determined whether this ssH2B is part of the histones, which remain in the mature mouse sperm (O'Brien and Bellvé, 1980). Since the analyzed cDNA clone did not represent a complete mRNA, the N-terminal end of the putative ssH2B protein is not known. The presence of tetrapeptide extensions in the 5` region comparable to those in SpH1 and SpH2B of sea urchins can therefore not be excluded.

A completely different point of view on the function of testis-specific histones, especially of TH2A and TH2B is posed by the failure to find testis-specific electrophoretic variants of H2A and H2B in several mammalian species (Meistrich *et al.* 1985). These authors suggested that mainly histone turnover during meiotic prophase might be essential while the testis-specific histone variants have no unique functions. The altering of the regulatory or promoter region of one of the multiple copies of H2A and H2B genes during evolution might have induced their specific expression in spermatocytes. Such a gene might subsequently have mutated into a testis-specific histone.

### *Spermatogenesis in* Drosophila

Although the studies on spermatogenesis in *Drosophila* were extensive (reviewed by Lindsley and Tokuyasu 1980; Hennig 1985; Hackstein 1987; Lifschytz 1987; Hennig 1989; Hennig *et al.* 1989; Hennig and Kremer 1990) little attention had been given to chromatin reorganization. Cytochemical data of Das and coworkers (1964) and Hauschteck-Jungen and Hartl (1982) suggest a transition from lysine- rich to arginine-rich chromosomal proteins coincident with the condensation of the chromatin, late in spermiogenesis. A substitution of histones at that stage is also suggested for *Drosophila hydei* where, as in *D. melanogaster*, the chromatin becomes highly compacted in sperm. A nucleosomal organization of this sperm chromatin is very unlikely regarding the volume of the sperm nucleus together with the size of the haploid genome (Grond 1984).

Changes in the chromatin during spermatogenesis are not limited to those generally accompanying meiosis and the final chromatin condensation late in spermatogenesis. In primary spermatocytes no histone H1 could be detected in the chromatin with an antibody raised against against somatic H1. However, H1 could be detected in the chromatin of spermatogonia and very early primary spermatocytes (chapter II). Furthermore a reorganization of the chromatin, namely a retraction and subsequent dispersion, in early spermiogenic cells was suggested by Feulgen staining (Grond 1984). The nucleus of these early spermatids undergoes a series of characteristic changes. Protein bodies are developed. They appear for the first time in the secondary spermatocyte. The separation of early spermiogenesis in distinct stages by Grond (1984) is based on the changes in structure and number of these structures. Their function has not been resolved until now. Grond (1984) already proposed a role in chromatin organization because they appear mainly to be composed of basic proteins. Morphologically and structurally identical structures are present in nuclei of pole cells. There are, however, no indications for the function of protein bodies from this occurrence.

*Outline of this thesis*

Although histone transition has been studied in a variety of species resulting in information on the different basic nuclear proteins present during spermiogenesis and in mature sperm, data on the function of these proteins are scarce. The complexity of chromatin makes *in vitro* experiments difficult to be interpreted with respect to the *in vivo* situation.

*Drosophila* is a suitable system for studying functional aspects of histone transition. The screening for mutations offers the possibility for analysis of the function of histone transition. Furthermore, *D. melanogaster* P elements can now be used as molecular tools in mutagenesis, cloning and rescue experiments.

Described in this thesis are studies on the cytological and molecular aspects of histone transition in *Drosophila hydei*. The choice of this *Drosophila* species is in part based on its superior cytology of spermatogenic cells. A second reason was the reaction of a lampbrush loop with an antibody raised against histone H1 (chapter II).

The detailed analysis of chromatin organization during spermatogenesis of *D. hydei* was started on the cytological level using the DNA stain DAPI (4′,6-diamidino-2-phenylindole dichloride) (chapter II). The observed sequence of changes in chromatin organization together with immunofluorescence studies using antibodies directed against somatic histones of *D. melanogaster*, induced the molecular approach of histone transition in *D. hydei*.

A prerequisite for the analysis of histone transition during spermatogenesis is the knowledge of somatic histones. For this purpose the genes coding for the presumptive cell-cycle regulated somatic histones were cloned and characterized (Chapter III). The DNA sequences of the presumptive cell-cycle regulated genes were used as a probe for the analysis of histone transcripts in testis and somatic tissues. With these experiments the existence of histone variants in testis was indicated as is discussed in Chapter IV. In Chapter V the cloning of one of the H3 histone variant cDNAs is described. These data can form the basis for a more detailed ananlysis of histone variants in *Drosophila* spermatogenesis.

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Chapter II

# Chromatin organization in the male germ line of *Drosophila hydei*

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**Abstract.** The chromatin organization in developing germ cells of *Drosophila hydei* males was studied with the highly sensitive DNA-stain DAPI (4′,6-diamidino-2-phenylindol-dichloride). The prophase of meiosis I is characterized by decondensed chromosomes and only late during this stage do they condense rapidly. Sex chromosomes show allocycly. During postmeiotic development the final chromatin condensation is preceded by a cycle of condensation and subsequent decondensation of the chromatin. Meiotic chromosomes were studied in more detail after orcein-staining. Pairing sites of the sex chromosomes could be localized in the distal end of the heterochromatic arm of the X chromosome and distally in both arms of the Y chromosome. The various heterochromatic parts of the genome condense differentially in meiosis. Chromatin reorganization was studied cytochemically with antibodies raised against histones H1 and H2A of *D. melanogaster*. The core histone H2A is present in spermatid nuclei until the late elongation stages. However, histone H1 is not found in the chromatin later than the early primary spermatocyte stage. Thus chromatin reorganization during spermatogenesis in *D. hydei* is complex. The process is discussed with regard to possible functions.

## Introduction

Spermatogenesis in *Drosophila* has attracted attention since Morgan (1912) discovered that crossing over is rare or absent in males. After the discovery of Bridges (1916) that in X/O males spermatogenesis is incomplete, particular attention has been focussed on the function of the Y chromosome in this developmental process.

The Y chromosome is actively transcribed during the primary spermatocyte stage and this is accompanied by the formation of giant lampbrush loops. These loops are most prominent in species of the *hydei* subgroup (Hess and Meyer 1963a). It has been shown that the lampbrush loops are covered with huge transcripts (Grond *et al.* 1983; de Loos *et al.* 1984), however, their main constituents are proteins (Grond *et al.* 1984). Molecular studies of Y chromosomal DNA sequences derived from the loop pair nooses and threads have revealed no indication of the protein coding functions of the fertility genes (Huijser and Hennig 1986; Vogt and Hennig 1986a,b) These data, together with the results of cytological, immunological and biochemical observations have led to the hypothesis that the main function of the Y chromosomal fertility genes is accumulation of specific nuclear proteins (for details see Hennig 1985; Grond 1984; Hulsebos *et al.* 1984).

During spermatogenesis a cellular morphology is adapted capable for the transfer of the paternal genome into the egg. In many organisms this process is accompanied by a substitution of the normal chromosomal histones by protamines or other proteins more basic than somatic histones (for review see Bloch 1969, 1976; Chevaillier 1983; Subirana 1983). The result is tight packaging of the chromosomal DNA in the sperm nucleus. For *D. melanogaster* it has been shown that during chromatin condensation in postmeiotic stages of spermatogenesis (*i.e.* during spermiogenesis) arginine-rich proteins are substituted for lysine-rich nuclear proteins (Das *et al.* 1964; Hauschteck-Jungen and Hartl 1982). A pre-

liminary light microscopy study in spermatogenesis in *D. hydei* has indicated that chromatin reorganization is a complex process (Grond 1984). In this paper a more detailed study of the constitution of chromatin throughout meiosis is presented, based on the use of DAPI (4',6-diamidino-2-phenylindole dichloride) a highly sensitive fluorescent DNA dye which permits the detection of even small amounts of DNA and their location in the nucleus (Russell *et al.* 1975; Williamson and Fennell 1975).

Our results confirm and extend the observations of Grond (1984) on the complexity of chromatin reorganization during spermatogenesis. We studied chromosome condensation during meiotic prophase and observed the allocyclic behaviour of the sex chromosomes. Postmeiotic chromatin constitution is characterized by an initial condensation and decondensation cycle before the final condensation. These observations and recent molecular data on the Y chromosome in *D. hydei* are discussed with regard to the hypothesis that the X chromosome is inactive during the meiotic prophase.

## Materials and Methods

*Drosophila stocks. D. hydei* males from the wildtype and white stocks of our laboratory were grown at 24 °C.

*Preparation of DAPI-stained slides. Method I*: Testes of pupae or newly eclosed males were dissected in testis isolation buffer (0.183 M KCl, 47 mM NaCl, 10 mM Tris-HCl pH6.8; Hennig 1967). After puncturing with a needle the testes were gently squashed in a drop of testis isolation buffer on a microscope slide. Siliconized coverslips were used and were removed after freezing in liquid nitrogen. Slides were immediately transferred into 96% ethanol where they were kept until use (10 min - 2 h). Fixation was done in 3.7% formaldehyde in PBS (0.137 M NaCl; 2.7 mM KCl, 1.15 mM $KH_2PO_4$, 6.5 mM $Na_2HPO_2.2H_2O$, pH 7), for 10 min. After three washes in PBS (7 min each) and one wash (5 min) in Tris buffer (0.18 M Tris/HCl, pH7.5), the slides were stained for 10 min with DAPI (Boehringer; 0.5 µg/ml in Tris buffer). Staining was performed in a moist chamber in the dark and the DAPI solution was freshly diluted from a stock-solution (0.1 mg/ml DAPI in Tris buffer). The following steps were carried out in the dark. After one wash in Tris buffer (5 min) and three washes in PBS (7 min each) slides were mounted in PBS. Coverslips were sealed with nail polish and the slides were stored in the dark at 4°C.

*Method II*: Testis of pupae or adult males were dissected in testis isolation buffer and immediately transferred into the fixative (3% formaldehyde in testis isolation buffer). After fixation (1 h) the testis were washed twice in testis isolation buffer (7 min each) and once in Tris buffer (5 min). Staining was performed in the dark with 0.5 µg/ml DAPI in Tris buffer. After one wash in Tris buffer (5 min) and two washes in testis isolation buffer (7 min each) in the dark testis were gently squashed in a drop of testis isolation buffer on a slide. Coverslips were sealed and slides were stored in the dark at 4°C.

Method I was used for studying spermatogonia, primary spermatocytes and early and mid spermatids. Late stage IV spermatocytes, and the subsequent stages of meiosis as well as late spermatids were studied using method II. Photographs

of late spermatids were taken from unsquashed testes. Flies from a white stock were used because of the lack of pigmentation of the testis wall. Wild type testes are highly pigmented.

*Preparation of orcein-stained meiotic chromosomes.* After dissection in testis isolation buffer pupal testes were immediately transferred into a hypotonic solution of 0.95% sodium citrate and incubated for 5 min The testes were stained in 3% orceine in 70% acetic acid for 2 min. After a short rinse in 45% acetic acid testes were squashed in the same solution. Coverslips were sealed and the slides were immediately examined.

*Immunofluorescence.* Testes of *D. hydei* adult males were dissected in testis isolation buffer and gently squashed in a drop of the buffer. After freezing in liquid nitrogen coverslips were removed. The slides were immediately transferred into 96% ethanol where they were kept until use. Preparations were fixed in 3.7% formaldehyde in PBS for 12-15 min and subsequently in 3.7% formaldehyde in 45% acetic acid (3 - 5 min). After washing in PBS (4x7 min) the slides were transferred into 1% Triton X-100 in PBS (5-7 min). They were washed again in PBS (4x7 min). For reaction with the first antibody (see below) the slides were incubated in a moist chamber with 40 µl of the antiserum. After washing in PBS (4x7 min) the slides were incubated with the second antibody in a moist chamber in the dark. Before mounting in PBS slides were washed (4x7 min in PBS). Coverslips were sealed with nailpolish.

*Antibodies.* The anti-histone antibodies were kindly provided by Drs. H. Saumweber and M. Frasch (Max-Planck Institut für Entwicklungsbiologie Tübingen) and were raised against histones H1 and H2A of *D. melanogaster* embryos. The antiserum H1 no. 190 was polyclonal and raised in a rabbit; the antiserum against H2A (Bx91/6) was monoclonal. Sheep anti-rabbit fluorescin isothiocyanate(FITC)-labelled immunoglobuline (Miles Laboratories, Hamburg) and goat-anti mouse FITC labelled immunoglobuline (Nordic Immunological Laboratories, Tilburg) were used as second antibodies.

*Microscopy.* The slides were examined with a Zeiss photomicroscope III with epi-fluorescence equipment. An HBO 200 w/4 lamp was used for illumination with Zeiss filtercombinations BP365, FT395, LP397 for DAPI and BP455-490, FT510, LP520 for FITC. Photographs were taken on Agfapan 25 or 100 Professional film.

## Results

Earlier descriptions of *D. hydei* spermatogenesis concentrated on the morphology of the different stages (Meyer 1963; Hennig 1967; Hess and Meyer 1968; Grond *et al.* 1984). However, our investigation was on the constitution of the chromosomes and chromatin. The difficulties of such studies had been pointed out by earlier investigators (see Cooper 1950) but with the aid of fluorescence techniques, enabling us to detect even minor amounts of DNA in their location in the nucleus, we obtained a picture of the structural organization of the chromatin throughout spermatogenesis.

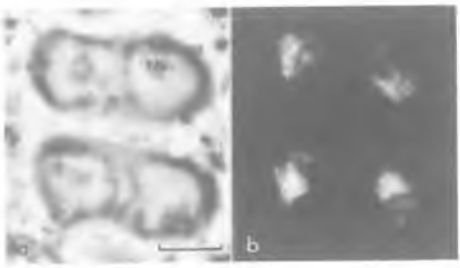

**Fig. 1.** Four spermatogonia. Coarse granular chromatin is dispersed in the nucleus. No chromocenter can be distinguished. **b** DAPI-staining by method I. Nu nucleolus. Bar represents 5 μm

*Premeiotic development*

In *Drosophila* spermatogonia are located in the apex of the testis (for review see Lindsley and Tokuyasu 1980). By mitosis secondary spermatogonia are continuously produced which develop within cysts, surrounded by two cyst cells. In interphase spermatogonia, the chromatin is dispersed all over the nucleus (Fig. 1) and the chromosomes are closely associated. A chromocenter can be distinguished in some of the cells and the chromatin has a coarse granular appearance. No separate heterochromatic centromere regions can be identified which may indicate that they are joined in the chromocenter together with the Y and the X chromosomal heterochromatin, or that they are masked by the granular structure of the chromatin. A third, although unlikely explanation is that kinetochore regions are negatively heteropyknotic in spermatogonia as they are during the first meiotic division (see below). Metaphase chromosomes are extremely rare and are morphologically comparable with metaphase chromosomes of neuroblasts.

*The meiotic prophase*

In the upper part of the testis tube the majority of the cells are in meiotic prophase.

Eight of such cells, called primary spermatocytes, develop synchroneously within one cyst. The primary spermatocyte phase can be subdivided into five stages (O-IV) (Hennig 1967). Stage O may be considered as the interphase (including the S-phase) of the spermatocyte and the structure of the chromatin cannot be distinguished from that in spermatogonia. The other 4 stages belong to meiotic prophase I and their nuclear cytology is characterized by the lampbrush loops developing from the Y chromosome.

Spermatocyte stage I is characterized by an increase in nuclear volume and by the first signs of development of the Y chromosomal lamprush loops.
In phase contrast and by DAPI staining (Fig. 2a-c) it can be seen that the individual bivalents move independently from the nucleolus towards the periphery of the nucleus opposite the nucleolus. The separate clusters of chromatin can only represent the paired bivalents since their number - four - corresponds to the number of large autosomes. Apparently, the fifth pair of autosomes, the dots, moves in most cases in close association with one of the four larger bivalents and is only occasionally seen separated.

During their movement the autosomes start to decondense. The chromatin becomes more spread out and the coarse granular structure changes to a finely granular one. Gradually the appearance becomes reminiscent of the typical chromomeric pattern of meiotic prophase chromosomes. Heterochromatic centromere regions cannot be discerned throughout prophase.

The Y chromosome has nucleolus organizer regions (NORs) at both chromosome ends (Meyer and Hennig 1974; Hennig *et al.* 1975) and remains attached to the nucleolus. In early spermatocytes the heterochromatic Y chromosome is associated with the X heterochromatin (Fig. 2a-c). The DAPI fluorescence in Y heterochromatin is stronger than that in the heterochromatic arm of the X chromosome. During spermatocyte stage I the Y chromosome starts, like the other chromosomes, to decondense and the first indications of the lampbrush loops can be detected. The decondensation of the Y chromosome does not seem to be a continuous process. Initially, at least three condensed blocks remain visible which are connected by thin, fluorescent threads. These blocks gradually decrease in size and disappear in the course of spermatocyte stage I.

The chromosomal material which is associated with the Y chromosome in the region close to the nucleolus, has been interpreted as the X chromosome (Meyer 1963, Hennig 1967, Yamasaki 1977, Grond *et al.* 1984, see also Hennig 1985). This could be further substantiated by our analysis of DAPI-stained spermatocytes of X/O males. The X chromosome is attached to the nucleolus with its heterochromatic arm which carries a NOR (van Breugel 1970; Hennig *et al.* 1975), hence it is distinctly separated from the autosomes. The euchromatic arm of the X chromosome resembles the autosomes (Fig. 2a,b and, more prominent, Fig. 2d,e), *i.e.* it is decondensed and is granular. An unequivocal identification of the heterochromatic arm was possible by comparing DAPI-stained wildtype spermatocytes with spermatocytes of males lacking most of the X heterochromatin.

The increase in cellular volume during spermatocyte stages 0 and I is accompanied by an increase in the number of mitochondria (Grond *et al.* 1984). The DAPI fluorescence of the mitochondrial DNA is detectable throughout all stages of spermatogenesis (Figs. 2-4, 6, 7). Only in late spermatids and in spermatozoa can the mitochondrial DNA not be detected any more.

In primary spermatocyte stage II the lampbrush loops are fully developed (Fig. 2d-f). The Y chromosome is extremely decondensed (with the occasional exception of a small block attached to the pseudonucleolus) and is not granular like the other chromosomes. Thus even chromosome regions not clearly carrying genetic complementation groups (see Hennig 1985) must be highly decondensed. This is consistent with the model of wholly despiralized chromatids as proposed by Hennig (1967). With DAPI fluorescence it was possible for the first time to follow the DNA axis in some of the loops (Ns, Cl, Th; Fig. 2d,f). It appears as a uniformely thick fluorescent string inside the nooses and threads and as a coiled string of decreasing diameter in the clubs, in agreement with earlier models of the lampbrush loops. No evidence for chromomeres or other condensed chromatin blocks was found. It is obvious that those loop regions refractive in phase contrast do not have a higher DNA content than others. Sometimes, in the tubular ribbons (Tr) a dotted staining could be detected which might be an indication of some local coiling of the DNA.

Decondensation of the autosomes continues during spermatocyte stage II during which they are located at the periphery of the pear-shaped nucleus, opposite to the nucleolus. At the end of stage II, when the nucleolus starts to

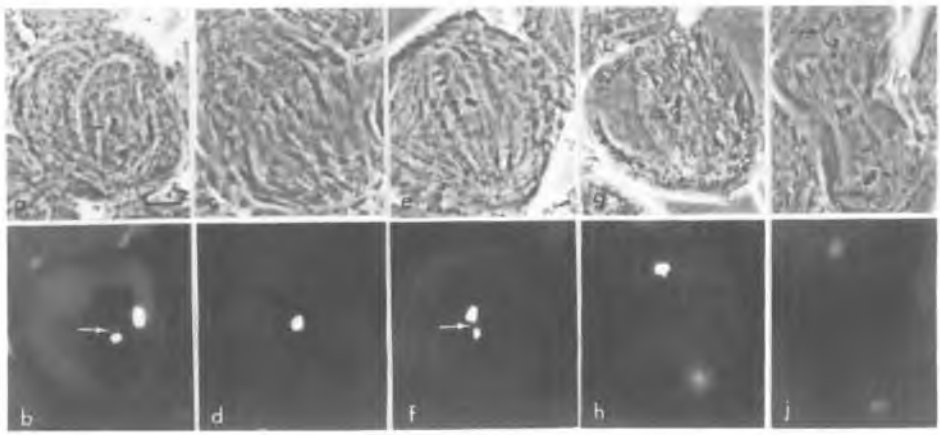

**Fig. 3.** Meiosis I. **a,b** prometaphase I. Fine chromatin threads extend from the condensed chromosomes (*arrow*). Mitochondria in the spindle show fluorescence. **c,d** Metaphase I. Chromosomes are located at the equatorial plate. **e,f** Early anaphase I. The autosomal kinetochore-associated heterochromatin is negatively heterpyknotic (Fig. 5). (*arrow*). **g, h** Late anaphase I. Chromosomes are less condensed. The heteropyknosis of kinetochore associated heterochromatin has disappeared. **i, j** Telophase I. Two nuclei are formed (*arrow*). The chromatin is homogeneously stained. The residal structures of the lampbrush loops are located outside the nuclear compartment. DAPI-staining according method II. *Small arrows* in a, c and e residual material of lampbrush loops. Bar represents 10 μm

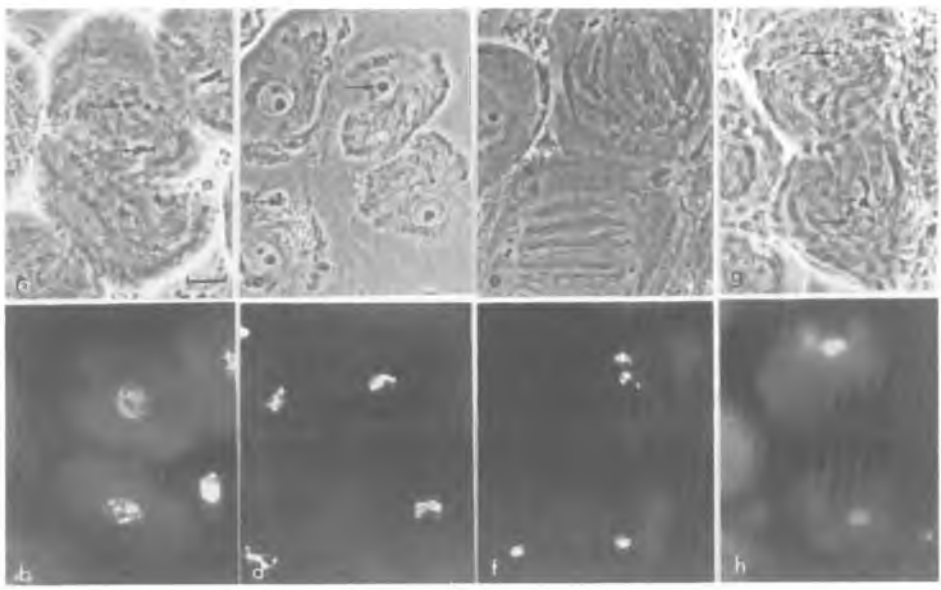

**Fig. 4.** Meisosis II. **a, b** Early secondary spermatocyte. The chromatin has a reticular structure due to the onset of condensation. Protein bodies (*large arrows*) are developing in the nucleus. **c, d** Late secondary spermatocytes. The chromatin forms an irregularly condensed mass. Large protein bodies are present in the nuclei. **e, f** Early

1950, p. 35). However, the metaphase chromosomes in *D. hydei* clearly show a normal equatorial arrangement during the first meiotic metaphase.

During metaphase I the extremely condensed chromosomes are located in the equatorial plate of the nuclear compartment (Fig. 3c,d). The chromosomes are closely associated and are hardly recognized as separate entities. Comparable to the situation in spermatocyte stage IV, in some metaphases I thin chromatin strings extend from the chromosomes. Irregular structures with low refractivity structures as described for spermatocyte stage IV are found within the nuclear compartment. They contain no DNA detectable by DAPI-staining (Fig. 3).

At anaphase I negative heteropyknosis of the autosomal kinetochore-associated heterochromatin becomes visible (see below). These chromosome regions are directed towards the spindle poles (Fig. 3e,f). In the DAPI-stained anaphases it was not possible to distinguish the sex chromosomes because of the high degree of chromosomal condensation and the intense fluorescence. The chromosomes move simultaneously to the poles, and just before their arrival the negative heteropyknosis of the kinetochore regions has disappeared and the chromosomes start to decondense (Fig. 3g,h). During telophase I the newly formed nuclei are filled with homogeneously stained chromatin (Fig. 3i,j). The boundaries of the spindle-shaped nuclear compartment as seen during meta- and anaphase I have disappeared and two small irregular shaped nuclei are formed. The remnants of the loops are located outside these nuclei. In contrast to earlier stages, the material is now highly refractive in phase contrast. The number and sizes of the structures are still variable.

The secondary spermatocyte can be identified by its two asters and the single centrioles (Grond 1984). After the nuclei have increased in volume the chromatin starts again to condense and a fine network appears (Fig. 4a,b) which gradually becomes coarser. An irregular, partially condensed chromatin mass develops (Fig. 4c,d). Chromosomes are not discernably separated and as was described for metaphase I, less condensed chromatin threads extend from the extremely condensed chromatin mass. One to four nucleolus-like structures, called protein bodies (Grond 1984), develop within the nuclei at the beginning condensation of the chromatin. They showed no DAPI fluorescence and appear to be devoid of DNA. During condensation of the chromatin the protein bodies increase in size (Fig. 4c) and when the condensation is almost complete only one large protein body is found in the nucleus. In metaphase II the location of chromosomes in the equatorial plate is comparable with that in meiosis I. Anaphase II differs from anaphase I by the lack of negative heteropyknosis of the kinetochore regions which is not found in anaphase II (Fig. 4e,f). During anaphase II the chromosomes start to decondense, but earlier than in meiosis I and no protein bodies can be recognized with the nuclear compartment which, just as in the first meiotic division, is surrounded by parafusorial membranes (Grond 1984).

As soon as the new nuclear membrane is formed, the chromatin appears homogeneously decondensed (Fig. 4g,h). One or two very small protein bodies develop in the nuclei, one of which increases in size and forms a large protein body.

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**(upper) and late (lower) anaphase II. g, h Telophase II. Daughter nuclei with homogeneously stained chromatin are formed. One or two small protein bodies (large arrow) have developed. Fluorescence in the spindles is due to mitochondrial DNA. a, b, e-h DAPI-staining by method II; c, d staining by method I. *Small arrows* Residual material of lampbrush loops. Bar represents 10 μm**

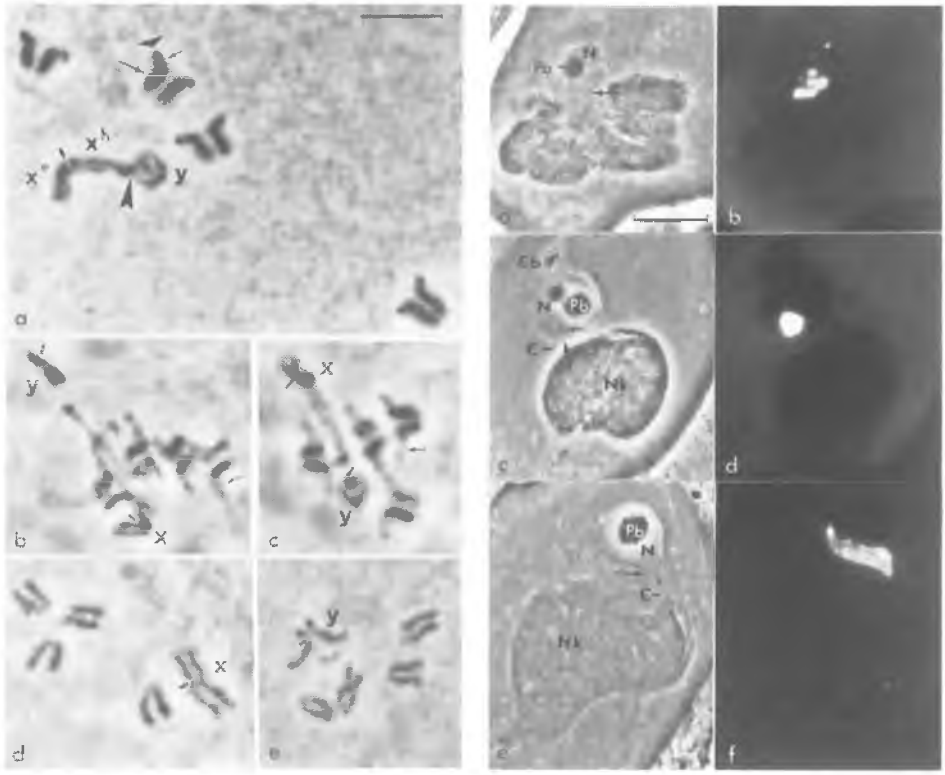


**Fig. 5.** Meiotic chromosomes, orcein-stained. **a** Late metaphase I. The kinetochore associated heterochromatin of the long arm of the autosomes is slightly negatively heteropyknotic (*small arrow*). The short heterochromatic arms (*short arrowhead*) and the kinetochore regions of the autosomes show repulsion. The euchromatic arms of the autosomes (*large arrow*) are paired. The proximal part of the X chromosomal heterochromatic arm is negatively heteropyknotic. The heterochromatic arm of the X is distally paired with the tip of both arms of the Y chromosome (*long arrowhead*). **b, c** Early anaphase I. The negative heteropyknosis of the autosomal kinetochore-associated heterochromatin and of the X heterochromatin is more prominent than in metaphase I (*small arrow*). Either the long arm (b) or the short arm (c) of the Y chromosome point into the direction of the X heterochromatin. **d, e** Metaphase II with an X (d) or Y chromosome (e). No heteropyknotic regions can be discriminated. Xe euchromatin of the X chromosome; Xh heterochromatin of the X chromosome. *Small bars* kinetochore of the X and Y chromosome. Bar represents 5 μm

**Fig. 6.** Postmeiotic development: pre-elongation stages. The chromatin condenses and subsequently starts to decondense. The mitochondria fuse and form the Nebenkern. **a, b** PMI. The chromatin is partly condensed and retracted to the fenestrated side of the nucleus. One protein body is present in the nucleus. **c, d** PMII. The chromatin is condensed near the attachement side of the centriole. Two large protein bodies and protein body-like structures (*small arrow*) in the chromatin region, are present in the nucleus. **e, f** PMIII. The chromatin is partly decondensed and spread along the fenestrated side of the nucleus. The protein body is covered with small granules. DAPI-staining according method I. C centriole; Ch chromatoid body; N nucleus; Nk Nebenkern; Pb protein body; *large arrow* fenestrated side. Bar represents 10 μm

In anaphase and telophase II the residual material of the loops is usually found within the spindle (Fig. 4e,g). During cytokinesis the material becomes unequally divided between the two daughter cells. At the end of telophase II one compact structure, called chromatoid body by Grond (1984), is found in the vicinity of the nucleus and sometimes an additional small round structure is present.

*Orcein-staining.* For highly condensed chromosomes the use of fluorescent dyes is not suitable because of restricted resolution. Therefore, we also studied the meiotic stages after hypotonic treatment and orcein staining of chromosomes. This is particularly suitable for the chromosomes of the first meiotic meta- and anaphases.

In metaphase I the euchromatic long arms of the autosomes, the distal part of the heterochromatic arm of the X chromosome (probably the region distal to the NOR, *cf.* Bonaccorsi *et al.* 1981) and the Y chromosome are extremely condensed (Fig. 5a). The kinetochore-associated heterochromatin of the long arm of the autosomes, however, is less condensed. During late metaphase (fig. 5a), the chromatids of the long arms of the autosomes, of Y and of both chromosome arms of the X chromosome are separated but not the short arms of the autosomes or of the Y chromosome. The euchromatic arms of the autosomes are still paired, but the short heterochromatic arms and the centromere regions show repulsion. Although earlier stages of the first metaphase could not be analysed because of the close association of the chromosomes, it is likely that the chromosomes at these stages are entirely paired.

During metaphase I the physical association of X and Y is closer than that of the homologous autosomes since both sex chromosomes are attached to another. The pairing region of the X chromosome is at the distal end of the heterochromatic arm but for the Y chromosome the situation is less clear. In metaphase a circular configuration of the Y was regularly found (Fig. 5a) and both ends of the Y chromosome are attached to the X chromosome. This may reflect the situation in the primary spermatocytes where the sex chromosomes are closely associated to one another (see Fig. 2). In early anaphase I either the short or the long arm of the Y or both arms point in the direction of the X (Fig. 5b,c). Pairing of the X and Y is interrupted as soon as the chromosome movement towards the poles starts, because of the short length of the paired region. Until late anaphase I the chromosome structure remains comparable to that of metaphase I. However, the negative heteropyknosis becomes more prominent during anaphase. This may be a consequence of the chromosome movement stretching the less condensed chromosome regions more strongly.

In metaphase II no clear differentiation in the chromosomes is seen after orcein-staining (Fig. 5d,e). The regions which showed heteropyknosis in meiosis I do not do so during meiosis II. The chromatids of the chromosomes are fully separated. As has already been described for DAPI-staining, decondensation of the chromosomes is seen before the chromosomes arrived at the poles.

### *Postmeiotic development*

The postmeiotic development of the nuclei was studied after DAPI-staining using the staging discribed by Grond (1984): PM I-Pm III, pre-elongation; PM IV-PM

V, elongation; PM VI, transition; PM VII, postelongation; PM VIII, individualization and coiling.

During PM I, the stage of aggregation of the Nebenkern, the nucleus and the larger protein body inside increase in size (Fig. 6a,b). The fine chromatin network which develops after telophase II, becomes coarser and the chromatin retracts to one side of the nucleus. This is probably the fenestrated side identified in EM studies (Grond 1984) since the nuclear envelope is more refractive in phase contrast (Fig. 6a). Irregularly sized chromatin masses develop and mitochondria fuse to form the Nebenkern but the mitochondrial DNA can still be identified by its fluorescence.

At the end of PM I and the onset of PM II (Nebenkern stage) chromatin forms a highly compact, irregular mass, which comprises only a minor proportion of the nuclear volume (Fig. 6c,d). It is always found at the place where the centriole attaches to the nuclear membrane. Two or three protein bodies (or similar structures) of low refractivity are located in the center of the chromatin.

Stage PM II nuclei can be identified by the presence of several protein bodies (Fig. 6c). During this stage the chromatin starts to decondense and splits into several blocks interconnected by thinner threads, which spread along the fenestrated side of the nuclear membrane. However, it is not possible to recognize individual chromosomes. In some nuclei we observed an association between the chromatin and one of the larger protein bodies.

The decondensation and spreading of the chromatin continues during stage PM III (separation of the Nebenkern into its two components; Fig. 6e,f) and within it condensed and more diffuse portions can be seen. The chromatin is restricted to the region of the nucleus close to the fenestrated side and does not extend into regions of the nucleus above the protein bodies. In early PM III thin chromatin threads between the chromatin and the protein body are sometimes observed. The single protein body of PM III spermatids is typically covered with small granules. Mitochondrial DNA can be recognized in the ovoid Nebenkern which starts to separate into its derivatives (Fig. 7a,b).

Stage PM IV is characterized by the beginning of elongation of the nucleus and the Nebenkern derivatives. The fenestrated part of the nuclear membrane now extends from the apex of the nucleus to the attachment site of the centriole. The chromatin becomes distributed all over the nucleus and in parts of the region close to the fenestrated side of the nuclear membrane it is more condensed than in other regions.

The homogeneous distribution of chromatin found in early PM V is maintained during this stage (Fig. 7c,d). The diameter of the nucleus changes in the course of elongation and decreases more strongly between the middle of its length and the beginning of the flagellum. The DNA content, indicated by DAPI fluorescence, in this region is lower than in the residual part of the nucleus (Fig. 8a,b). However, at the attachment site of the flagellum a strongly fluorescent dot is present which is not correlated with the mitochondrial DNA fluorescence in the Nebenkern derivatives. It is first recognized during stage PM V, but is continuously present until stage PM VIII.

During the subsequent development the size and shape of the nucleus as seen in the light microscope does initially not change. Then the chromatin condenses and the nucleus aquires its final needle-like shape (Fig. 8c).

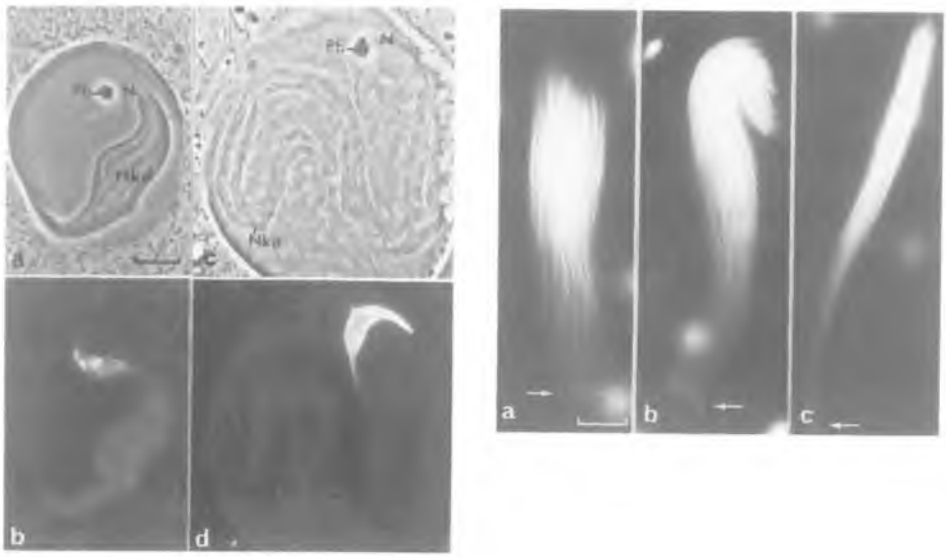


**Fig. 7.** Postmeiotic development: early and mid elongation stages. **a, b** PMIV. The chromatin is spread all over the elongating nucleus. The distribution of the chromatin is not homogeneous. The small granules attached to the protein body have fused. **c, d** PMV. The chromatin is homogeneously distributed all over the nucleus. One protein body covered with a few large grana is present in the nucleus. Mitochondrial DNA shows a dotted fluorescence in the Nebenkern derivates. DAPI-staining according method I. N nucleus; Nkd Nebenkernderivates; Pb protein body. Bar represents 10 μm

**Fig. 8.** Postmeiotic development: elongation and post-elongation stages. The shape of the nucleus changes during elongation. **a** PMV. Nuclei of a cyst with elongating spermatids. The diameter of the nucleus decreases between the middle and distal end. This region shows only a low fluorescence. A strongly fluorescing dot (*arrow*) is present at the attachment site of nucleus to the flagellum. **b** Spermatid nuclei after elongation. Their form and the distribution of the chromatin is as in figure 8a. **c** Nuclei of spermatids immediately before coiling, showing the final needle shape. The distribution of the chromatin is as in figure 8a,b. The fluorescing dot (*arrow*) is present at the attachment site of the nucleus to the flagellum. Bar represents 10 μm

*Immunofuorescence*

The observations on the chromatin constitution during spermatogenesis encouraged us to investigate the presence of somatic histones in the various stages of germ cell development by immunofluorescence. We used antibodies raised against the somatic histone H1 from embryos (polyclonal) and against the core histone H2A (monoclonal) of *D. melanogaster*. The cross-reaction of these antibodies with *D. hydei* somatic histones was confirmed in polytene chromosomes and testis wall nuclei.

*Histone H2A.* The core histone H2A can be visualized in chromatin from the spermatogonial stage through meiosis until late elongation (Fig. 9) the fluorescence pattern is identical to that obtained with DAPI.

In spermatogonia and in stage O primary spermatocytes fluorescence is dispersed in the nucleus (Fig. 9a,b). As with DAPI-staining, a chromocenter can

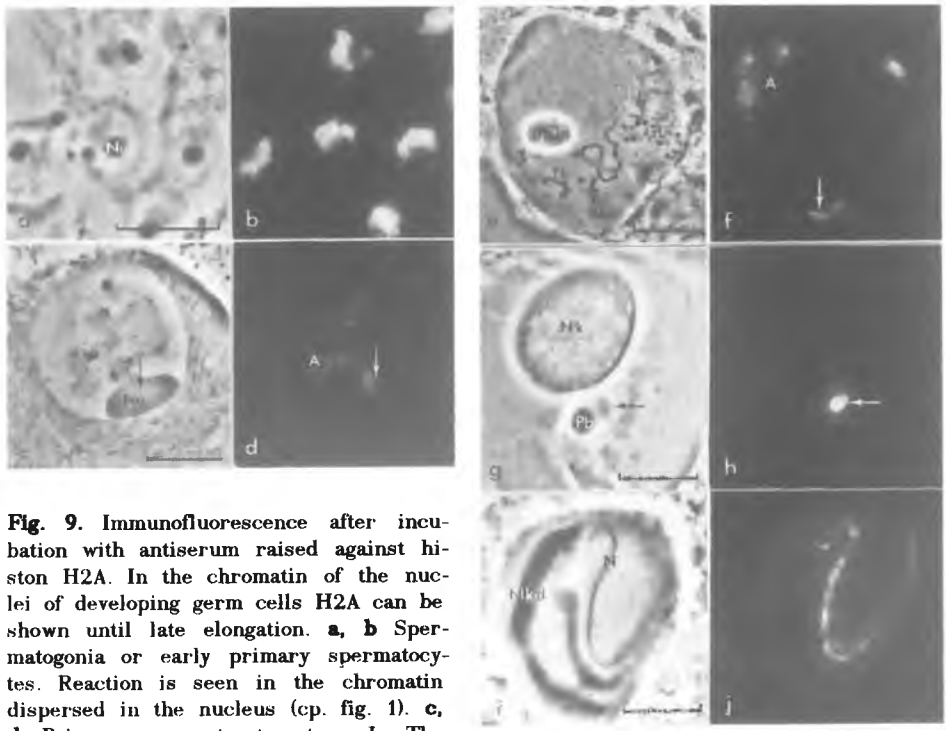


**Fig. 9.** Immunofluorescence after incubation with antiserum raised against histon H2A. In the chromatin of the nuclei of developing germ cells H2A can be shown until late elongation. **a, b** Spermatogonia or early primary spermatocytes. Reaction is seen in the chromatin dispersed in the nucleus (cp. fig. 1). **c, d** Primary spermatocyte stage I. The chromosomes which move to the periphery of the nucleus and the chromatin attached to the nucleolus, the X and probably the decondensing Y chromosome, show a positive reaction (cp. fig. 2a-c). **e, f** Primary spermatocyte stage II. The autosomes and the X chromosome (*arrow*) are fluorescent (cp. fig. 2d-f). **g, h** PMI. The chromatin which is strongly condensed in this stage, shows a positive reaction, with the antibodies against H2A (cp. fig. 6b). **i, j** PMV. Reaction is seen in the nucleus of the elongating spermatid. A Autosomes; Cl clubs; N nucleus; Nk Nebenkern; Nkd Nebenkern derivatives. Nu nucleolus; Pb Protein body; Ps pseudonucleolus; Th threads. Bars represent 10 μm

sometimes be discriminated. In stage I primary spermatocytes, H2A can be shown in the autosomes, the nucleolus, in the X chromosome and probably in the decondensing Y chromosome (fig. 9 c,d). The autosomes and the X chromosome (euchromatin and heterochromatin) also show fluorescence in stage II primary spermatocytes. No reaction could be detected in the Y chromosome (Fig. 9 e,f).

From the various stages of the meiotic divisions we only found metaphases in our preparations but because of the positive reaction with antibodies against H2A during metaphase and immediately after meiosis, it is likely that H2A is present throughout meiosis. H2A can also be shown in the chromatin in early postmeiotic development (Fig. 9 g,h) and until late elongation a positive reaction is found in the nuclei of spermatids (Fig. 9 i,j). At the end of stage PM V and later in spermiogenesis H2A can not be shown which might indicate substitution or masking of this protein.

*Histone H1*. In contrast to core histone H2A the presence of somatic histone H1 can only be shown in the premeiotic development and in very early prophases of meiosis I. (This is independent of the fixation conditions: when the fixation

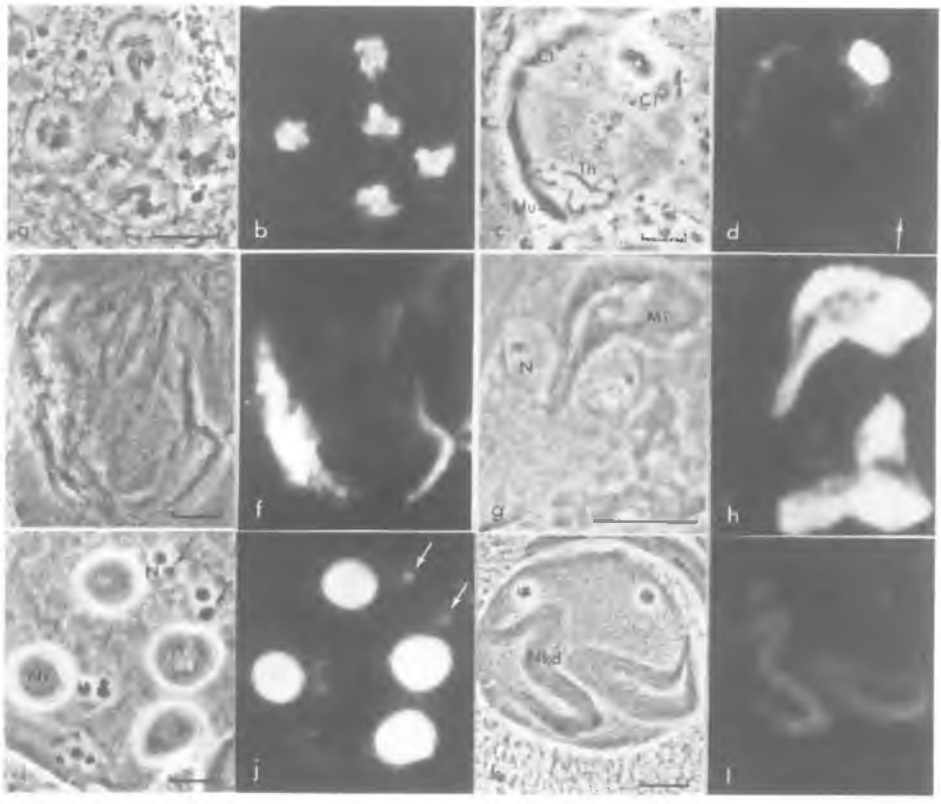


**Fig. 10.** Immunofluorescence after incubation with an antiserum raised against histone H1. Only in spermatogonia and young primary spermatocytes of stage O the presence of H1 can be detected in the chromatin. **a, b** Spermatogonia or early secondary spermatocytes. The dispersed chromatin shows fluorescence (cp. figs. 1 and 9a, b). **c, d** Primary spermatocyte stage II. Antigens are found in the pseudonucleolus and the grana of the clubs. No fluorescence is found in the chromatin (*cf.* figs. 2d-f and 9d-f). Also the mitochondria show fluorescence (*arrow*). e, f Meiotic divisions I and II. In the spindles a strong reaction occurs, which is probably related to the mitochondria. Within the nuclear compartment no reaction is seen. **g, h** PMI. A strong fluorescence is seen in the aggregating mitochondria, whereas the nuclei display no reaction. **i, j** PMII. A positive reaction occurs in one of the protein bodies (*arrow*). The mitochondria have fused and formed the Nebenkern, which also displays a strong reaction. **k, l** PMV. The reaction in the protein body and the Nebenkern as established in PMII is maintained. Cl clubs; N nucleus; Nk Nebenkern; Nkd Nebenkern derivatives; Nu nucleolus; Pb protein bodies; Sp spindle; Th threads. Bar represents 10 μm

step in 3.7% formaldehyde in 45% acetic acid is omitted the pattern of reaction with the anti-H1 serum does not change).

In spermatogonia and early primary spermatocytes of stage 0 the chromatin react positively with antiserum to H1 (Fig. 10 a,b). This reaction is comparable with that after DAPI-staining (*cf.* fig. 1). As the nuclear volume increases the reaction in the chromatin disappears and thereafter, no H1 can be shown in the chromatin (Fig. 10 c-f). However a strong reaction is seen in the lampbrush loops

pseudonucleolus and clubs (grana) of stage II-IV primary spermatocytes (Fig. 10 c,d) (*cf.* Fig. 9e,f and Fig. 2d,f). During meiosis no immunofluorescence is seen in the nuclear compartment (Fig. 10 e,f). The same holds true for early postmeiotic development (PMI, fig. 10 g,h). However in PM II one of the protein bodies shows a positive reaction with the antiserum. This reaction can still be seen in the single protein body of stage PM IV (Fig. 10i-l).

The strong immunofluorescence in the mitochondria of primary spermatocytes and in the meiotic spindle is notable (Fig. 10 c-f) and continues after aggregation of mitochrondria into a Nebenkern (Fig. 10 g-l). However, it decreases in the course of elongation (Fig. 10 k,l). Fluorescence in the mitochondria is likely to be due to cross-reaction of the antibody with other mitochrondrial proteins since no histones have so far been detected in mitochondria. Caron *et al.* (1979) describe the presence of a lysine-rich histone-related, DNA-binding protein.

## Discussion

The chromosome structure during spermatogenesis of *Drosophila* has been extensively studied (see Cooper 1950) but classic methods of chromosome fixation and staining have not been very suitable to investigate meiotic chromosomes. The use of fluorescent dyes and a modified fixation procedure allowed us to obtain a more detailed picture of the chromosome constitution during the meiotic phase in the male, the criterium for preservation of a good chromosome morphology being the cytology of the lampbrush loops in primary spermatocytes. Two periods during spermatogenesis were shown to merit special attention. Firstly, as already stated by Cooper (1950), the chromatin condensation is abnormal, and does not occur in the usual sequence of meiotic prophase chromosome condensation. The allocyclic behaviour of the sex chromosomes, and, in particular, the complete decondensation of both sex chromosomes which has been questioned before (see below) were observed. Secondly, in early postmeiotic development, we observed an extra cycle of chromosomal condensation and decondensation. These two aspects and additional observations will be discussed in more detail.

### *Meiotic prophase I*

The course of the meiotic prophase I in *D. hydei* is rather different from that in most other organisms and of the chromosome morphology characteristic of the conventional phases cannot be detected. The chromosomes disperse early in the primary spermatocyte stage and maintain the dispersed state throughout the entire meiotic prophase I. Then, before the first meiotic division, condensation occurs which must be very fast since configurations intermediate between the dispersed state and metaphase chromosomes are rarely observed. The only relationship to typical meiotic prophase chromosomes is the granular appearance of the autosomes and of the X chromosome, which is simular to that of chromosomes carrying chromomeres.

Decondensation of chromatin is generally considered as an indication of transcriptional activity but this may not be true of primary spermatocytes. At the onset of spermatocyte stage III the level of RNA synthesis decreases strongly

(Hennig 1967: Fig. 7). Nevertheless, the highest degree of decondensation of the autosomes and of the euchromatic part of the X is only found during spermatocyte stages III-IV (see Fig. 2h,k). Also the high degree of decondensation of the loop forming part of the Y chromosome is maintained until spermatocyte stage IV. Spermatocyte stages III and IV last approximately 24 h (Hennig 1967). Such an extended period of high chromatin decondensation might have other biological reasons than transcription. The cytology of the lampbrush loops in stage III spermatocyte nuclei cannot be explained by the cessation of initiation of RNA synthesis since in this case a gradual decrease in loop size and gradual condensation of the chromatin during stage III should be expected. It must be recalled that the Y chromosomal lampbrush loops during the primary spermatocyte stage are densely covered with giant transcripts (Grond *et al.* 1983; de Loos *et al.* 1984), which, together with associated proteins (Grond *et al.* 1984), produce the typical loop morphology . The time required for the complete transcription of a single loop has been calculated to 7 - 20 hours, depending on the particular loop (see Grond *et al.* 1983; de Loos *et al.* 1984). The maintenance of the loop structure suggests, therefore, that the growing transcripts may not be completed by moving all along the DNA of the loop but rather that they are gradually detached from their DNA matrix during stage III or IV. If this occurs randomly along the loop it could result in an essentially unchanged structure throughout stage III , even without transcriptional activity. A new set of chromosomal proteins may become bound to the DNA inhibiting further attachment of the RNA polymerase or the detachment of polymerases at this stage could facilitate gradual association of the loop DNA with chromosomal proteins required for the successive condensation of the chromosomes.

A typical element of chromatin structure is the nucleosome, but nucleosomes are rare in highly transcribed DNA regions. This has also been demonstrated for the Y chromosomal loop DNA (Grond *et al.* 1983; de Loos *et al.* 1984). The presence of core histones in the autosomes throughout meiotic prophase and in all chromatin of the subsequent stages of meiosis was demonstrated with the aid of histone antisera raised against somatic histone H2A. By electron microscopy nucleosomes have been shown in the primary spermatocyte chromatin. In contrast, histone H1 thought to cause condensation of inactive chromatin, has only been detected in early primary spermatocytes and is absent from the chromatin of any of the subsequent meiotic or postmeiotic stages. Our failure to detect histone H1 by immunofluorescence microscopy could be due to a masking effect at these developmental stages. On the other hand, it has been demonstrated for various organisms that during the first meiotic prophase germ line specific histone H1 variants are present which probably replace the somatic H1. For example, in rat spermatogenesis testis-specific histones are synthesized (Meistrich *et al.* 1985). Thus, the transcriptional inactivity of the decondensed chromosomes in spermatocyte stage III may be necessary for the assembly of germ line-specific histones and, possibly, other chromosomal proteins in the chromosomes.

Reorganization of chromosomal proteins in the primary spermatocyte stage of *D. melanogaster* has also been indicated by the cytochemical studies of Hauschteck-Jungen and Hartl (1982). After staining with Brilliant Sulfo Flavine no reaction was found in primary spermatocytes but in other stages basic proteins could be detected. Similar observations have been made for *Laspeyresia pomonella* (Lepidoptera) (Friedländer and Hauschteck-Jungen 1982).

*Heterochromatin during meiosis*

In *D. hydei* at least 20% of the genomic DNA is localized in heterochromatic chromosome regions (Hennig *et al.* 1972). During meiosis heterochromatin can, however, only be recognized during restricted periods. In the decondensed state of the chromosomes in the spermatocyte stage, the autosomal heterochromatin is in distinguishable from euchromatin. The X heterochromatin and the Y chromosome, however, behave allocyclically. The heterochromatin of the X chromosome remains in a condensed state much longer than that of other chromosome regions and its decondensation is restricted to a limited period towards the end of spermatocyte stage II. The condensation of the sex chromosomes at the end of the prophase begins earlier than for the autosomes. This positive heteropyknosis of the sex chromosomes during meiotic prophase I contrasts with the negative heteropyknosis of a major part of the heterochromatic arm of the X chromosome during metaphase I and anaphase I. The autosomal kinetochore-associated heterochromatin behaves similarly. In all other stages of meiosis no differential condensation of chromosome regions can be recognized.

Allocyclic behaviour has also been described for the sex chromosomes of *D. melanogaster* by Cooper (1950). He also observed that at least in "early" primary spermatocytes of *D. melanogaster* no heterochromatic elements are detectable (Cooper 1959: p. 566). Our preliminary studies of DAPI-staining in *D. melanogaster* spermatocytes also revealed a transient decondensation of at least part of the X chromosome (Kremer, unpublished). The observation that heteropyknotic elements are absent during parts of the spermatocyte stage in *D. melanogaster* and *D. hydei* is contradictory to the results and the model of Lifschytz and Lindsley (1972) which postulates X chromosome inactivation in primary spermatocytes of all species with heterogametic males as a prerequisite for male germ cell development. Our data, however, clearly prove that during a considerable period of the first meiotic prophase the euchromatin of the X chromosome is entirely decondensed and even the heterochromatic arm becomes decondensed transiently. The cytological and ultrastructural morphology of the X chromosome euchromatin is similar to the cytology and ultrastructure of the autosomes (see also Grond *et al.* 1984). Moreover, autoradiographic studies of $^{3}$H-uridine incorporation in RNA in primary spermatocytes of X/O males clearly demonstrated the transcriptional activity of the X chromosome (Hennig 1967, Fig 9) The unequivocal localization of the X chromosome by the DAPI-staining substantiates the assignation of the label close to the nucleolus in X/O males to the X chromosome (Hennig 1967). Thus, X chromosomal loci are transcribed during the primary spermatocyte stage.

*Meiotic pairing of the sex chromosomes*

In *D. hydei* we never observed a close association of major parts of the sex chromosomes in spermatocytes during prometaphase I and subsequent meiotic stages. In prophase I the relationship between X and Y chromosomes could not be determined as both chromosomes are closely associated with the nucleolus but the high degree of decondensation of the loop forming part of the Y chromosome in particular must exclude extensive pairing during this period. During metaphase I one or both ends of the Y chromosome are usually associa-

ted with the end of the heterochromatic arm of the X chromosome. In *D. melanogaster* also the pairing is restricted to limited regions of both sex chromosomes, called collochores (Cooper 1950, 1964). Two such regions are located in the proximal heterochromatin of the X chromosome and in the Y chromosome, one is found in the short and the other in the long arm, both close to the kinetochore.

There is little evidence for DNA homology of the regions of meiotic pairing of the sex chromosomes in *D. melanogaster* or in *D. hydei*. Although both the X heterochromatin and both arms of the Y chromosome in *D. hydei* carry nucleolus organizer regions. This region in the X chromosome is not terminally located (van Breugel 1970; Hennig *et al.* 1975). Hybridization experiments have also not provided evidence for homology of the terminal regions of the X and Y chromosomes (Hennig *et al.* 1983; Huijser and Hennig 1986). Thus, the role of homologous sequences in pairing between the sex chromosomes in *Drosophila* is unclear although in mouse and man there is clear evidence for non-homologous pairing of the sex chromosomes (for review see Ashley 1984).

### *Secondary spermatocytes*

The interphase between the meiotic telophase I and prophase II is very short, since only a few secondary spermatocytes with decondensed chromatin were observed. Protein bodies are formed from the onset of condensation which must be considered as meiotic prophase II. For several *Drosophila* species, cells with a reticular chromatin and nucleolus-like bodies have previously been described as interphase II (Cooper 1950; Meyer 1963; Liebrich 1981). But this is probably incorrect since the appearance of the protein bodies seems to indicate the beginning of chromatin condensation.

### *Postmeiotic chromatin organization*

We have shown that postmeiotic chromatin undergoes an extra cycle of condensation and decondensation before it is finally packed densely into the sperm head. The biological role of this cycle is unclear but it is probably correlated with the molecular reorganization of the chromatin. A complete substitution of the histones cannot take place at this stage, since core histones (H2A) are still found during spermatid elongation. However, histone H1 is not detectable by immunofluorescence and is either absent or masked in postmeiotic stages or has been substituted by H1 variants with other immunological properties or by other chromosomal proteins.

In *Achaeta domesticus* changes in chromatin organization were observed before an alteration in the content of somatic histones had occured (Kaye *et al.* 1978). Since the nucleosomes were still present conformational changes must have been mediated by other chromosomal proteins. Posttranslational modification of histones was not found (Kaye *et al.* 1978). In *D. hydei* the immunological data suggest that somatic histones are present but that H1 behaves differently from the core histones. Thus, the change in chromatin conformation might be mediated by changes in nonhistone chromosomal proteins as well as H1.

The appearance of arginine-rich proteins late in spermatid development

seems to be rather common in insects, for example, in *A. domesticus* (Kaye and McMaster-Kaye 1966; McMaster-Kaye and Kaye 1976) and in *Laspeyresia pomonelly* (Friedländer and Hauschteck-Jungen 1982b). For *D. melanogaster* a transition from lysine-rich proteins to arginine-rich chromosomal proteins has been detected cytochemically (Das *et al.* 1964; Hauschteck-Jungen and Hartl 1982). During elongation almost no basic nuclear proteins were found by Hauschteck-Jungen and Hartl (1982), in contrast to the results of Das *et al.* (1964). Staining characteristic of arginine-rich proteins occured with increasing intensity during and after the final chromatin condensation in spermatid nuclei (Hauschteck-Jungen and Hartl 1982).

Olivieri and Olivieri (1965) and Cooper (1950) described the preferential localization of chromatin close to the fenestrated side of the nucleus in the early stages of *D. melanogaster*. This chromatin, however, is not condensed to the same extent as in the first chromatin condensation in *D. hydei* (see also Tates 1971). Our preliminary study on *D. melanogaster* indicates that the first condensation cycle is not as extreme as in *D. hydei*.

In spite of all these observations little evidence on the possible role of the first condensation/decondensation cycle in *D. hydei* spermatid nuclei is available. To our knowledge the occurrence of such an event has not been reported before and therefore, it is difficult to relate other observations to our data. An understanding of the chromatin organization during the postmeiotic phase can only be achieved by techniques other than cytology.

### *The protein bodies*

The protein bodies are remarkable structures in the nuclei of secondary spermatocytes and spermatids. In earlier descriptions of the spermatogenesis of *Drosophila* they were assumed to be nucleoli but, this was later excluded (for discussion see Tates 1971; Lindsley and Tokuyasu 1980). The protein bodies undergo a characteristic and probably biologically relevant sequence of changes in number, structure and size, strictly correlated with the developmental stage of the cell (see also Grond 1984).

By light and electron microscopy it has been shown that the protein bodies contain basic proteins (Grond, 1984; Kremer, unpublished data). One component may be histone H1 or an immunologically related protein since the protein bodies in spermatid nuclei react with H1 antiserum. The amount of nucleic acids in the protein bodies must be minute, (Grond 1984) and no DNA could be detected with DAPI-staining although the protein bodies were often were closely associated with chromatin. Autoradiographic studies of $^{3}$H-uridine-labelled testes gave no evidence for the presence of RNA but it may be present in the small granules attached to the protein bodies in spermatid stages PM II-V (Grond 1984).

Protein bodies have also been observed and studied in other organisms, in particular in spermatid nuclei of *Achaeta domesticus* (Kaye and McMaster-Kaye 1966). Where nuclear inclusions appear in early spermatids. They vary in number, size and structure and are composed of proteins; weak staining with Azur B may indicate RNA (Kaye and McMaster-Kaye 1966).

The changes in number and size of the protein bodies occur in parallel with changes in the degree of condensation of the chromatin. This, together

with the basic nature of the proteins in the protein bodies and their possible relationship to histones or histone variants, makes it reasonable to postulate a role in the chromatin organization during and after meiosis. Experimental evidence to support this idea has been obtained from studies of the postmeiotic chromatin organization of X/O males of *D. hydei* (Kremer, unpublished data) in which an aberrant organization of the protein bodies is seen and the first postmeiotic condensation of the chromatin is disturbed. In addition, the chromatin never becomes fully condensed in elongating spermatids of X/O males. Grond (1984) has suggested that the protein bodies function as a reservoir of chromosomal proteins during chromatin reorganization. The presence of histone H1 in the protein body of certain postmeiotic stages supports such an idea.

That the actual role of the protein bodies might be more complex is indicated by the characteristic changes occurring during the postmeiotic spermatid development. In particular, the amount of material in the protein bodies of *D. hydei* is high compared with the amount of chromosomal proteins which would be necessary for packaging the genome. This is concluded from the size and compactnes of the protein bodies (Grond 1984) and from the volume of condensed chromatin in stage PM II. The large amount of material in the protein bodies in *D. hydei* is emphasized by a comparison of the size of the protein bodies in the spermatid nuclei of *D. hydei* and *D. melanogaster*. It could be argued that substitution of histones requires an excess of the substituting protein but this is difficult to reconcile with the differing between the two species: the volume of the protein bodies, differs by 10- to 50-fold although the difference in the genome sizes is approximately 1.7-fold (Mulder *et al.* 1968; Rasch *et al.* 1971).

The difference in the volumes of the protein bodies may be related to the different sizes of the Y chromosomal lampbrush loops in the primary spermatocytes and here also much more protein is accumulated in *D. hydei* than in *D. melanogaster*. There seems to be a correlation between the protein bodies and the lampbrush loops in spermatocytes. H1 antiserum shows a positive reaction with lampbrush loop pseudonucleolus and the grana of the clubs in the primary spermatocytes. After meiosis, a reaction is found only in the protein bodies. In addition, Glätzer (1984) has shown that RNA-associated proteins occur in spermatocyte nuclei, associated with several of the lampbrush loops, and can later be found in the protein bodies. Thus, several proteins have been discovered which accumulate in the nuclear compartment before the meiotic divisions and are found postmeiotically within the nuclei. Although the results of Glätzer (1984) are compatible with the model of protein storage in the fertility genes, it is less likely, although not impossible, that RNA-associated proteins (see also Risau *et al.* 1983) are involved in chromatin reorganization. Risau *et al.* (1983) suggest that these hnRNA-associated proteins have RNA processing functions. The presence of hnRNA-associated proteins in the Y chromosomal fertility genes and in the spermatid nuclei during some postmeiotic stages may be related to observation (Hennig, 1968; Hennig *et al.*, unpublished) that Y chromosomal RNA fractions are still found in spermatids. Possibly nuclear RNP is gradually released to the cytoplasm of the spermatids and regulatory effects at the translational level could take place (see Hulsebos *et al.* 1983; Brand *et al.*, in preparation). Since the genome is not transcribed during postmeiotic development, regulatory mechanisms other than at the transcriptional level are necessary in spermatid differentiation and protein bodies may be invol-

ved. The potential presence of RNA in the granules attached to the protein bodies (Grond 1984) supports this hypothesis as does the observation that the size difference of the protein bodies between *D. hydei* and *D. melanogaster* parallels the size difference of the spermatozoa. In *D. hydei* the length of a spermatozoon exceeds 10 mm (see Grond 1984), while in *D. melanogaster* it is approximately 1.8 mm (Cooper 1950; Hess and Meyer 1963b; Meyer 1968). Therefore, the amount of material required for such a structure must be much higher in *D. hydei* and the larger protein bodies could be related to this requirement.

*General aspects of chromatin reorganization*

Our study suggests that several peculiarities in the chromosome structure during and after meiosis are related to the reorganization of the chromatin. Such a reorganization seems to be characteristic of the development of the male gametes. The details of chromatin organization are not well understood and the relevance of changes is unclear. It is usually assumed that chromatin reorganization serves to protect the genome within the sperm nucleus against detrimental external influences after leaving the male genital tract. An alternative possibility is the suppression of transcription. However, it has not often been considered, that the onset of the early development requires the reprogramming of the paternal and maternal genomes (cf. Hennig 1986). Spermatogenesis represents a highly sophisticated differentiation process, which must certainly be regulated at the genomic level of the male germ cells. The genome must therefore be subject to reprogramming in the embryo in contrast to the situation in almost all other cell types achieving a high degree of cellular differentiation. In these cases the cells are not reprogrammed but reach a terminal state of differentiation. At the earliest, reprogramming of the male germ cells could occur during the late prophase I of meiosis since the primary spermatocyte stage is responsible for the production of the material required for postmeiotic spermiogenesis (*cf.* Hennig 1985). We observed that during late meiotic prophase I (spermatocyte stage III/IV) the transcriptional activity of the genome had ceased while it remained in a decondensed state for an extended period. We suppose that this period is required for a first cycle of substitution of chromosomal proteins.

In the literature on chromatin reorganization during spermatogenesis also the phenomenon of imprinting, which is characteristically observed in some organisms, has not received much attention. Reprogramming of the genome and imprinting are different phenomena, but they may be coupled during spermatogenesis and related to the extensive chromatin rearrangements which occur. Imprinting is usually considered as a rather specialized and atypical phenomenon. However, the recent experiments of Solter *et al.* (1985) have demonstrated that for normal early development of mouse embryos the presence of the paternal and the maternal genome is essential. This implies that programming of the respective genomes, necessarily involving imprinting, must have taken place. For reasons mentioned above such impriting would probably not have occurred earlier than the late first meiotic prophase. Our observations that several successive steps of chromatin reorganization occur in the development of the male germ cells of *D. hydei* may be of relevance for such processes.

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Chapter III

# Isolation and characterization of a *Drosophila hydei* histone DNA repeat unit

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**Abstract.** Histone genes in *D. hydei* are organized in tandemly repeated clusters, accomodating in total 120-140 repeat units. We cloned one of the repeat units and analysed the nucleotide sequence. The repeat unit has a size of 5.1 x $10^3$ base-pairs and contains one copy of each of the genes coding for the core histones and one gene copy coding for the histone H1. In the promoter regions of the genes we identified the presumptive cap sites and TATA boxes. In addition we identified two sequence elements shared by all five *Drosophila hydei* histone genes in the cluster. The sequence CCCTCT/G is found in the region upstream of the presumptive CAP sites. The sequence element AGTGAA occurs downstream of the presumptive cap sites and is, in contrast to the promoter element, also seen in the histone genes of *Drosophila melanogaster* (Matsuo and Yamazaki 1989). Cell-cycle dependent regulation of transcription of the *Drosophila* histone genes may be different from that in other eukaryotes since sequence elements involved in the regulation of cell-cycle dependent transcription are absent. Also other regulatory elements for transcription differ from those of other histone genes. The highly conserved H1-specific promoter sequence AAACACA and the H2B specific promoter sequence ATITGCAT, which are involved in the cell-cycle dependent transcription of those histone genes in eukaryotes, are missing in the *Drosophila* genes. However at the 3′ end of the genes the palindrome and the purine-rich region, both conserved sequence elements in histone genes of eukaryotes, are present. The spacer regions show a simple sequence organization. The silent site substitution rate between the coding regions of the *D. hydei* and *D. melanogaster* histone genes is at least 1.5 times higher for *Drosophila* than for sea urchin histone genes.

## Introduction

The genes coding for the highly conserved histones constitute a multigene family. Their gene structure and organization in the genome have been studied in a variety of species. In higher eukaryotes histone genes generally occur clustered in the genome (for review see Maxson *et al.* 1983). Two types of clustering have been identified. In the first type the genes are arranged in random clusters dispersed throughout the genome. Examples are chicken (Engel and Dodgson 1981), mouse (Sittman *et al.* 1981) and man (Heintz *et al.* 1981; Sierra *et al.* 1982) In the second type of arrangement histone genes are organized as tandemly repeated clusters with each cluster containing all genes for the core histones and for H1. This has been found, for example, for the genes coding for the early histones in sea urchin (for review see Kedes 1979), *Nothophthalmus* (Stephenson *et al.* 1981) and *Drosophila* (Lifton *et al.* 1977)

In *Drosophila melanogaster* histone gene organization is known in detail. The majority of the histone genes, about 100 copies, exist in two types of repeat units called L (5.0 kb) and S (4.8 kb). They are localized in region 39 D-E of chromosome 2 (Lifton*et al.* 1977; Pardue *et al.* 1977). The two types of clusters differ by an insertion of a 240 bp tRNA-derived element in the H1 - H3 spacer region of the L unit (Lifton *et al.* 1977; Matsuo and Yamazaki 1989). Most of the two repeat types occur not intermingled (Saigo *et al.* 1980). In addition to the tandem repeats, orphon genes of H3 and H2B may be present (Childs et al. 1981).

In *Drosophila virilis* histone gene organization seems to be different from that in *D. melanogaster*. There are indications for a type of cluster without an H1 gene (Domier *et al.* 1986). Furthermore, histone genes are located at two different sites as shown by in situ hybridization on polytene chromosomes (Anderson and Lengyel 1983).

We started the analysis of the *D. hydei* histone genes since we have evidence for testis-specific histone variants (Kremer *et al.* 1986, and unpublished data). We cloned a 5.1 kb histone repeat unit from an Eco RI genomic library. A 5.1 kb EcoRI band is one of the main restriction fragments arising after hybridization with a *D. melanogaster* histone gene probe.

The 5.1 kb Eco RI fragment was sequenced. It contains the genes coding for the core histones and H1. Sequence analyses of coding regions, promoter regions and the remaining spacer regions including comparisons to the recently published sequence of a *D. melanogaster* repeat unit of the L type (Matsuo and Yamazaki 1989) are presented. The results of our comparison of the DNA sequences of both species allow to conclude that some of the regulatory elements for transcription of the *Drosophila* histone genes differ from those of other eukaryotes.

## Materials and methods

*Drosophila strains.*. The wild-type strain of *D. hydei* from our laboratory collection was used.

*Isolation of clone pDhH6/7.* A genomic library of *D. hydei* wild type DNA partially digested with EcoRI and cloned in vector λ641 was probed with nick-translated DNA of clone p604 (Chernyshev *et al.* 1980) containing the genes coding for H1 and the core histones of *D. melanogaster*. One of the selected *D. hydei* clones was designated λDhH6/7 and studied in detail. The 5.1 kb insert of this clone was recloned in pBR328 and designated as pDhH6/7. Screening, subcloning and isolation of recombinant DNA were performed according to protocols of Maniatis *et al.* (1982).

*DNA seqencing.* Cloning of selected as well as random restriction fragments of insert DNA of the clone pDhH6/7 in M13mp18-19 (Messing and Vieira 1982; Yanish-Perron *et al.* 1985) and isolation of single-stranded templates were performed according to the Amersham protocols (1984). The nucleotide sequences were determined by the dideoxy chain termination method of Sanger *et al.* (1977). In the last steps of sequencing oligonucleotide primers synthesized according to known parts of the sequence were used. Parts of the sequence were determined from denatured M13 RF DNA of the subclones using T7 DNA polymerase according to protocols of Pharmacia. The DNA sequence was determined for both strands.

*DNA sequence analysis.* For assembly of gel readings the computer program of Staden (1982) was used. The GCG sequence analysis software package version 5 1987 (University of Wisconsin, Biotechnology Center) was used for further sequence analyses.

## Results and discussion

### *Structure and organization of the histone gene repeat unit*

The *D. hydei* histone genes mainly occur in tandemly organized repeat units of 5.1 kb in lengths as is shown in Bam HI digeste genomic DNA hybridized with pDhH6/7 insert DNA (Fig. 1, lane 1). EcoRI digested genomic DNA (Fig. 1, lane 2) shows heterogeneity within the repeats. The 5.1 kb band represents one of the main bands. The 2.3 kb and 2.9 kb fragments most likely together form one repeat unit comparable to the unit represented in clone pDhH6/7. By Southern blot analysis it was determined that H1 coding sequences are present in both fragments, H2A and H2B coding sequences in the 2.3 kb fragment and coding sequences of H3 and H4 in the 2.9 kb fragment. This is in agreement with the organization of genes in clone pDhH6/7 (Fig. 2, see below). Moreover the genomic clone pDhH6/8 with an insert of 2.3 kb is in its restriction pattern (Bam HI, HhaI and PstI) identical to the region of pDhH6/7 containing the genes H2A, H2B and the 3′ region of H1.

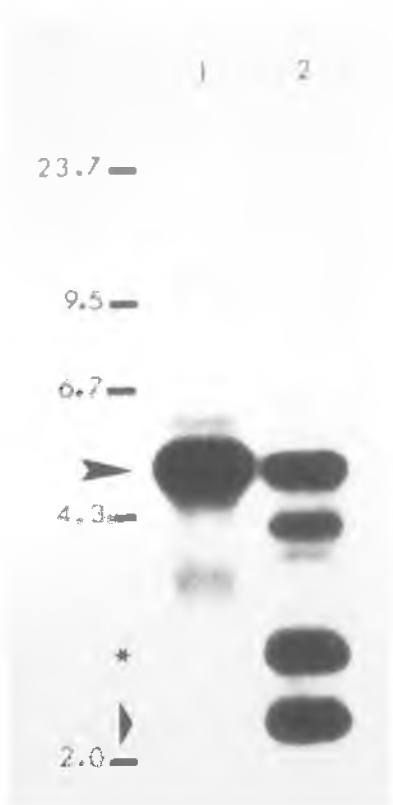



**Fig. 1. Hybridization of pDhH6/7 insert DNA to blots of BamH1- (lane 1) and EcoRI- (lane 2) digested genomic DNA of male flies. With genomic DNA of female flies the same results were obtained (data not shown). The molecular weight marker was HindIII-digested lambda DNA. *large arrowhead* 5.1 kb; *, 2.9 kb; *small arrowhead,* 2.3 kb**

The minor bands (Fig. 1) might represent orphan genes or genes coding for histone variants (van Daal *et al.* 1988) located outside the gene cluster (*cf.* Childs *et al.* 1981), repeat units with either a deletion or with an inserted transposable element (*cf.* Domier *et al.* 1986; Matsuo and Yamazaki 1989) or fragments at the border of the tandemly arranged repeat cluster.

The copy number of the histone gene cluster per haploid genome is determined to range between 120 and 140. By *in situ* hybridization it was determined that the cluster is located on chromosome 3 in region 62A2/3. By mistake a location in band 50A has been mentioned in Kremer and Hennig (1990).

A copy number of 5-10 and a location on chromosome 4 region 80C3/5 are given by Fitch *et al.* (1990). However, analysis of unpublished pictures of Fitch *et al.* showed that their location must be due to a different identification of the locus and not to a difference in location of the histone gene cluster. The difference in copy number probably is due to a different experimental procedure. The intensity of the hybridization signal in our *in situ* hybridizations makes a copy number of 5-10 very unlikely.

The general structure of the *D. hydei* histone repeat unit present in clone

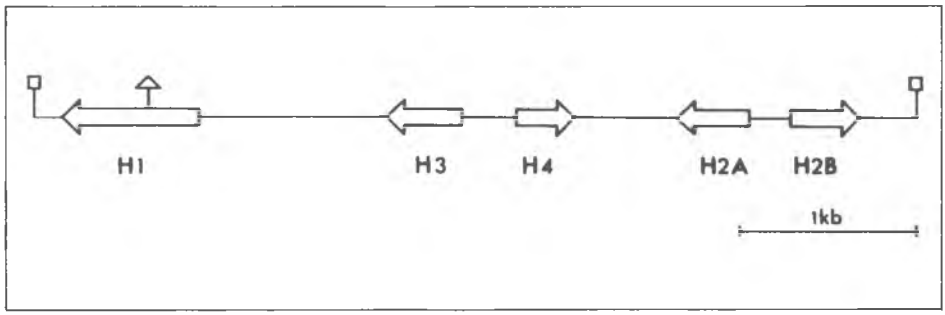



**Fig. 2. Organization of the histone genes within the repeat unit pDhH6/7. The direction of transcription is given by the *arrows* marking genes. BamH1, ⚲; EcoR1, ⚲**

pDhH6/7 is shown in figure 2. The total length of the *D. hydei* repeat unit is 5153 bp (base-pairs). The sequence is presented in Figure 3. After sequencing, the genes were located with aid of the *D. melanogaster* sequence of an L repeat unit (Matsuo and Yamazaki 1989). The arrangement and orientation of the genes within the cluster is the same as in *D. melanogaster* (Lifton *et al.* 1977; Matsuo and Yamazaki 1989).

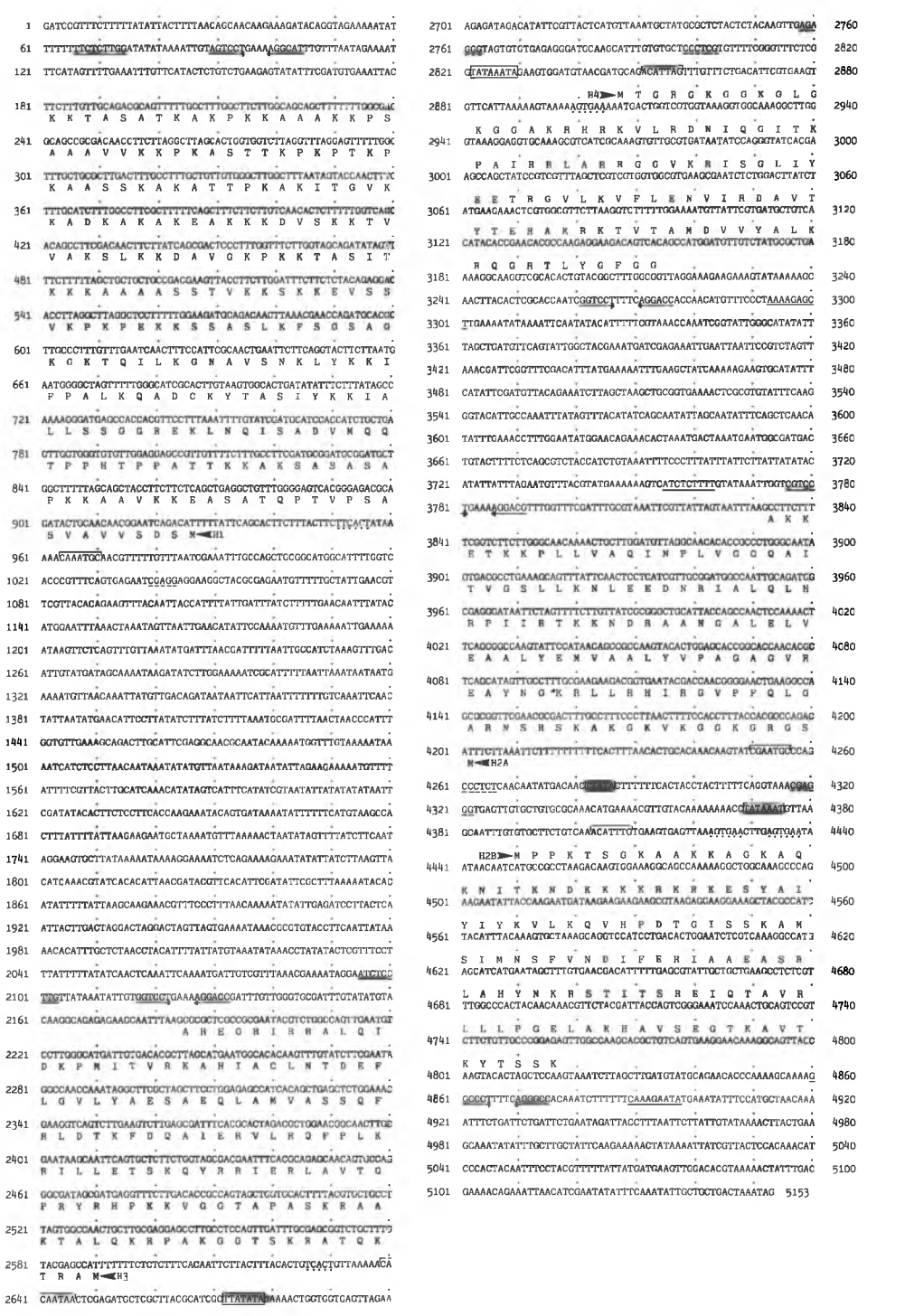
*Upstream sequence elements*

The 5′ upstream regions of the histone genes were examined for sequence elements relevant for regulation and initiation of transcription using literature data for upstream sequence elements of histone genes.

*Cap site and TATA box.* We searched upstream of the ATG codon for sequences similar to the consensus sequence for initiation of transcription PyAPyPyPyPy as defined by Breathnach and Chambon (1981) and to the cap site sequences of *Drosophila* genes given by Snyder *et al.* (1982) from which a consensus TCAGTT/C is deduced. Generally, the distance from the TATA box to the cap site is 25 to 30 bp in eukaryotic genes which are transcribed by RNA polymerase II (Breathnach and Chambon 1981). Taking into account this distance and the cap consensus sequences, the presumptive cap site of the H2B gene is located 40 bp upstream (pos. 4403,) of the ATG codon. A TATA box is present at -27 bp (pos. 4369).

Upstream of the H2A protein coding region two possible cap site similarities are found at -48 bp (pos. 4250) and -29 bp (pos. 4231) from the ATG. Because of its location 26 bp downstream of the TATA box (pos. 4282) the first of the two cap consensus sequences is the most likely candidate for the functional cap site. This is further supported by the occurrence of the same sequence GCATTCG in the *D. melanogaster* gene at the same distance from the TATA box (Matsuo and Yamazaki 1989).

**Fig. 3. Complete DNA sequence of clone pDhH6/7 and the deduced amino acid sequence of the histones. The start of the sequence is at the unique BamHI site in the H2B - H1 spacer region (see Fig. 4). The direction of transcription is marked with arrowheads. The following sequence elements are marked: ; cap site, ⌐¬ TATA box ▭; AGTGAA, ..... ; CCCTGT/G ---- ; palindrome, ← ; purine-rich region, ———**



For the H4 gene two possible cap sites are found at -50 (pos. 2851) and -54 bp (pos. 2847) from the ATG corresponding to a distance of 22 and 18 bp from the TATA box (pos. 2822). The presumptive cap site in *D. melanogaster* provides no argument in favour of one of these sites since there is no sequence similarity. The similarity to the cap sites of H2A, H2B, H3 and H1 (see below) of *D. hydei* favors the sequence ACATTAG at -50 bp from the ATG codon as functional cap site.

For the H3 gene potential cap site sequences are found at -61 (pos. 2651), -50 (pos. 2640) and -31 (pos. 2621) bp from the ATG. The sequence at -50 bp is most likely the cap site because of its distance to the TATA box (-26 bp, pos. 2672) and a 100% similarity at the same distance from the TATA box in the *D. melanogaster* H3 upstream region. The other sites have no sequence similarity in *D. melanogaster* at the same distance from the TATA box (Matsuo and Yamazaki 1989).

The cap site of the H1 gene of *D. melanogaster* has been determined by $S_1$ mapping at -34 +/- 2 bp from the start codon (Murphy and Blumenfeld 1986). A homologous sequence of 6 bp is found in the *D. hydei* H1 upstream sequence at -38 bp from the ATG codon (pos. 964). A clear TATA box is not found within 150 bp upstream of this presumptive cap site. The TATA box given for the *D. melanogaster* H1 gene by Matsuo and Yamazaki (1989) is located at -82 bp from the cap site. It is not a common phenomenon that H1 genes lack a TATA box or have a TATA box at a significantly larger distance from the cap site (see Wells 1986; Lai and Childs 1988). In *D. hydei* no similar sequence is found in the respective region. Alternatively, a 5 bp sequence (GCAAA) (pos. 992) may serve as a TATA box. GCAAA occurs in the H1 promoter region of both *D. hydei* (-22 bp from the cap site) and *D. melanogaster* (-25 bp from the cap site).

The transcription initiation site of many histone genes is located within the consensus sequence PyCATTCPu (Busslinger *et al.* 1980; Hentschel *et al.* 1980; Sures *et al.* 1980). This also holds true for the most likely candidates for cap site sequences of *D. hydei* and *D. melanogaster* histone genes. The presumptive cap sites resemble this sequence better than the sequence consensus for the *D. melanogaster* cap sites as given above.

*CAT box.* The CCAAT box is assumed to represent a general sequence element required for genes transcribed by RNA polymerase II (Breathnach and Chambon 1981; Dynan and Tjian 1985). This also holds true for histone genes in many organisms with the exception of H4 genes (Hentschel and Birnstiel 1981; Perry *et al.* 1985; see also Wells 1986). Comprehensive computer analyses of eukaryotic promoter elements by Bucher and Trifonov (1988) suggest that the preferential occurrence of this element is -50 to -110 bp from the cap site and that 100% homology to the pentamer is essential. This is in agreement with mutagenesis studies (Graves *et al.* 1986). However, -50 to -110 bp relative to the presumptive transcription initiation sites of both *D. hydei* and *D. melanogaster* histone genes, there is no sequence element with 100% homology to CCAAT or its reversed complementary sequence. Allowing one mismatch, several elements are found in this region. We therefore examined the upstream sequences of several *D. melanogaster* genes: the cuticle genes (Snyder *et al.* 1982), heatshock genes (Ingolia and Craig 1981), the yellow locus (Geyer *et al.* 1986), yolk protein

genes (Hung and Wensink 1983) and the β2 tubuline genes of *D. melanogaster* and *D. hydei* (F. Michiels, personal communication). Only in gene I of the cuticle genes a 100% similarity to the CAT box was found -88 bp from the cap site. These results support the suggestion of Bucher and Trifonov that CCAAT might not be a common promoter element in insects. Furthermore *Drosophila* culture cells lack CCAAT binding factors (Santoro *et al.* 1988).

*Sp1 binding site.* Sp1 transcription factor binding site(s) (GGGCGG or GC box) are found in many histone genes and other genes (Dynan and Tjian 1985; Cole *et al.* 1986; Artishevsky *et al.* 1987). Allowing one mismatch a screening for this sequence element in both orientations, showed only one GC box homology at -187 from the ATG (pos. 4258) of the H2B gene. In *D. melanogaster* GC box similarities with one mismatch are seen both in the H2B - H2A spacer region as well as in the H3 - H4 spacer. 100% GC box similarity is found in the *Drosophila* genes mentioned in the description of the CAT box analysis. It is not very likely that the few sequence similarities found represent true Sp1 binding sites since all functional Sp1 binding regions contain one or more perfect copies of the GC box (Dynan and Tjian 1985). These results together with the lack of Sp1 in *Drosophila* culture cells (Santoro *et al.* 1988) suggest that Sp1 is not a general transcription factor in *Drosophila.*

*Promoter elements specific for histone genes.* In the 5' upstream regions of both H1 (Coles and Wells 1985) and H2B genes (Harvey *et al.* 1982) of a variety of species, gene-specific sequence elements are found (see also Wells 1986).

A H1-specific heptamer AAACACA with perfect sequence identity is found in most H1 histone genes (Coles and Wells 1985). However, in both *D. hydei* and *D. melanogaster* this heptamer is not present in the 5' region of the H1 gene. Allowing one mismatch a region of similarity to this element is found in *D. hydei* at -51 (pos. 977) and -136 bp (pos. 1062) from the ATG codon. Since similarity to the heptamer with one mismatch is also seen in the upstream regions of several of the core histone genes in both *D. hydei* and *D. melanogaster,* the significance of the heptamer element in the H1 upstream region has to be doubted.

As is the H1-specific heptamer sequence, also the H2B-specific octamer ATTTGCAT is well conserved from sea urchin to man (see Wells 1986). The octamer is not found in the H2B genes of *D. hydei* and *D. melanogaster* unless 4 and 3 mismatches respectively are allowed. It has been shown that mutation of two nucleotides in the element, which change its sequence into the octamer AGTTGAAT, eliminates its function in a human H2B gene (Labella *et al.* 1988). Therefore it is unlikely that *Drosophila* H2B genes contain a functional octamer sequence. Although the octamer sequence element, in its conserved form, is absent from the *Drosophila* H2B genes, there are factor(s) present in crude *Drosophila* embryo extracts which bind to this octamer (Perkins *et al.* 1988). These octamer binding factor(s) might be cell-type or stage specific in contrast to the OTF-1 factor which generally is present in mammalia (*cf.* Schreiber *et al.* (1988) for B cell-specific OTF).

Both the H1-specific as well as the H2B-specific element are assumed to be required for cell-cycle dependent regulation of transcription in human cells (Dalton and Wells 1988; Labella *et al.* 1988) The lack of these two elements in the corresponding *Drosophila* histone genes known so far, suggests that the factors involved in cell-cycle dependent regulation of transcription of histone

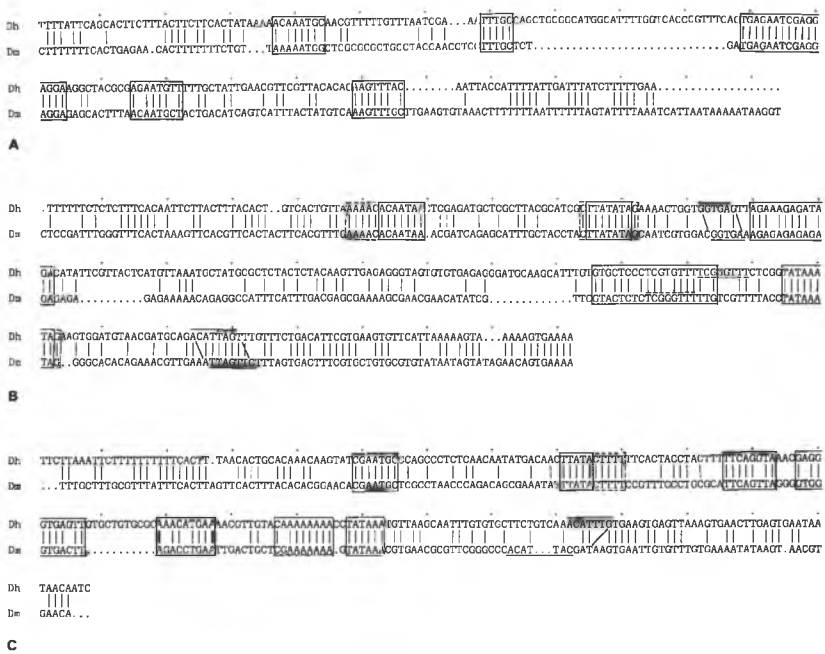


**Fig. 4. Alignment of the 5′ regions of the histone genes of *D. hydei* and *D. melanogaster*. Sequences were aligned using the program GAP of the GCG programs with a gap weight of 3.0 and a length weight of 0.2. For the 5' end of the H1 gene corrections were made. Cap site, TATA box and regions of similarity upstream of the cap site are marked. Sequences are presented in the same orientation as in figure 3. (a) 5′ region of H1 (204 bp, pos. 927-1130 in Fig. 3), (b) H3 - H4 spacer region (pos. 2591-2906 in fig. 3), (c) H2A - H2B spacer region (pos. 4203-4449 in fig. 3). Gaps are indicated by *dots*, identical nucleotides are marked by *dashes***

genes in *Drosophila* are different from those in a wide variety of eukaryotes.

In order to find promoter sequence elements shared by all five *D. hydei* histone genes we looked for perfect homologies of at least 5 bp in the 5′ upstream regions. One element (CCCTCT/G) occurs at -104 to -184 (pos. 1039, 2758, 2798, 4261 and 4317) from the ATG of all the *D. hydei* histone genes (see above). In *D. melanogaster* such an element is only found upstream of the H1 gene as part of the 16 bp conserved element (see Fig. 4). The functional significance of the CCCTCT/G element, if there is any, needs further experimental analysis.

### *Conserved sequence elements in the 3' region*

In the 3' non protein coding regions of cell-cycle regulated histone genes two conserved sequence blocks are found, namely a palindromic sequence and a purine-rich sequence 13 - 17 bp downstream of the palindrome. The palindrome (with a stem of 6 bp) is located in a region of about 24 bp (see Wells 1986), which is highly conserved from sea urchin to man (Busslinger *et al.* 1979; Wells 1986; for review see Birnstiel *et al.* 1985).

Both sequence elements are found in the *D. hydei* 3' downstream region of the histone genes (Fig. 3). The consensus sequence which can be deduced from the *D. hydei* and *D. melanogaster* sequences is conform to the consensus given by Wells (1986). The palindrome sequences of the H1 genes of both species are an exception between those of the core histone genes concerning the location of the inverted repeats, the imperfectness of the inverted repeats and divergence of its sequence between *D. hydei* and *D. melanogaster*. The distance of the repeat to the stop codon is significantly longer in H1 than in the core histone genes (73 bp in *D. hydei,* 85 bp in *D. melanogaster*). The distance in most histone genes given by Wells is about 30 bp with a maximum of 59 bp. Furthermore in both *D. melanogaster* and *D. hydei* the inverted repeat is imperfect which is an exception too (see Wells 1986). While the palindrome is perfectly conserved between the core histone genes of *D. melanogaster* and *D. hydei* for the H1 gene 3 out of 16 nucleotides are different.

The purine-rich region of the histone genes is well conserved between sea urchin species (consensus CAAGAAAGA) For vertebrate species only a loosely defined consensus sequence can be given (AAAAGAGCTG) (Birnstiel *et al.* 1985). Also among *Drosophila* species the purine-rich region is less well conserved than in sea urchin, thus resembling the situation in vertebrates. Since the purine-rich region, together with the U7 snRNA, is necessary for 3' end processing (Cotten *et al.* 1988), it would be worthwile to determine for the *Drosophila* U7 snRNA whether the larger sequence divergence in the purine-rich region finds its complement in a longer region of complementarity to the U7 snRNA as was found in mammals (Mowry and Steitz 1988; Soldati and Schümperli 1988).

For mouse histone H3 it has been shown that the palindrome sequence is involved in posttranscriptional, cell-cycle dependent regulation of the mRNA level. The conservation of the 3' downstream sequence elements suggests that this part of the cell-cycle dependent regulation of histone mRNA levels in *Drosophila* is not different from that in vertebrates. This is in contrast to sequence diversity in the elements involved in the regulation of transcription (see above).

In the histone gene sequences of *D. melanogaster* polyadenylation signals are found about 130 - 220 bp downstream of the translation stop signals (Matsuo and Yamazaki 1989). In *D. hydei* polyadenylation signals are only found downstream of the protein coding region for H2A (distance: 123 bp, pos. 3700) and for H3 (distance: 134 bp, pos. 2041). The suggestion of Matsuo and Yamazaki that *Drosophila* histone genes produce poly $(A)^+$ mRNA thus seems not likely for *D. hydei*. In particular, so far no poly $(A)^+$ histone mRNA is described for *Drosophila* with the exception of the H2AvD variant (van Daal *et al.* 1988).

### *Structure of the spacer regions*

The spacer regions of the histone repeat of *D. hydei* were screened for the occurrence of repeats, simple sequences and purine-pyrimidine stretches and they were compared with each other. The designation "spacer region" is used for those regions of the repeat unit not coding for proteins.

All spacer regions are AT-rich. Short (3-6 bases) homopolymers of dA or dT are regularly found, but rarely runs of dG or dC. In general, one can conclude

that the spacers show a rather simple sequence organization but that they are not internally repetitious. Analyses of the histone spacer sequences of *D. melanogaster* (Matuso and Yamazaki 1989) yielded the same picture.

The rather simple sequence organization of spacers and the occurrence of short homopolymers of dA or dT, or to a much lesser extent of dG or dC, is regularly observed in histone genes, for example in sea urchin (Schaffner *et al.* 1978; Sures *et al.* 1978; Roberts *et al.* 1984; Kaumeyer and Weinberg 1986) in *Xenopus* (Perry *et al.* 1985), trout (Winkfein *et al.* 1985), and in the sea star (Cool *et al.* 1988).

It is known that homopolymeric stretches of dA or dT have influence on the structure of DNA and in this way could affect nucleosomal positioning, DNA replication and transcription (Kunkel and Martinson 1981; Prunell 1982). However, the distribution and length of the homopolymers in *D. hydei* histone gene spacers is not comparable to that described for the promoter regions of the yeast His 3 or Pet 56 genes (Struhl 1985) or for a function in DNA bending (Snyder *et al.* 1986). A function of the poly (dA) and poly (dT) tracts of the *D. hydei* spacers in replication or in promoter functioning is therefore not very likely. They might however have a function in nucleosome positioning since homopolymeric runs longer than 4 or 5 bp have preferential rotational settings on the nucleosome supercoil (Satchwell *et al.* 1986).

Repetitive elements are frequent in the histone gene spacers of various organisms (Perry *et al.* 1985; Winkfein *et al.* 1985; Cool *et al.* 1988). This contrasts with the situation found in the *Drosophila* spacer regions of the histone genes, where no repetitious elements where found.

Another feature of histone gene spacers is the occurrence of short simple repeating sequence elements of two or three nucleotides in length (Sures *et al.* 1978; Perry *et al.* 1985; Winkfein *et al.* 1985 Tönjes and Doenecke 1989). However, in the *D. hydei* histone spacer regions no such elements with more than three repeats are present. The simple repeat of $(GA)_{10}$ of the H3 - H4 spacer of the histone repeat of *D. melanogaster* is not found in *D. hydei*, although the region in which the GA repeat starts is conserved between *D. melanogaster* and *D. hydei* (GAAAGAGA, pos. 2698, following a GT-rich region). The observation that the GA repeat is missing in the *D. hydei* H3 - H4 spacer in this case supports the proposal of Tautz and Renz (1984) that simple repetitive sequence elements in general have no specific function in gene expression. The differences which are generally found in histone gene clusters with regard to the occurrence of simple repetitive sequences (Schaffner *et al.* 1978; Sures *et al.* 1978; Perry *et al.* 1985) also favor this proposal.

The different spacers of the *D. hydei* histone repeat show no extensive homology with each other. This agrees with the situation in the *Xenopus* histone gene clusters (Perry *et al.* 1985). Comparison of the spacers by dot matrix analyses (window/stringency of 11/8) revealed only short similar sequence elements. The similarity close to the 5′and 3′ ends of the protein coding regions is somewhat higher than in other parts of the spacer. The leader regions between the presumptive cap sites and the ATG codons of the different histone genes seem to be constructed of homologous sequence elements. Their positions differ however in the different leaders. These elements are A/T-rich and often related to the sequence AGTGAA. AGTGAA elements itself are found once or twice in each of the leaders of the five histone genes in *D. hydei* (Fig. 3) as well as in

*D. melanogaster* (Matsuo and Yamazaki 1989). An exception is the H3 leader in *D. hydei* which shows only the sequence AGTGA. Matsuo and Yamazaki suggested a function of this element specific for *Drosophila* histone gene expression. This is supported by the conservation of this element in the histone genes of *D. hydei*.

At the 3' ends of the genes homology is restricted to the palindrome and the purine-rich region (see above).

Alternating purine-pyrimidine stretches longer than 6 bp are not found in the *D. hydei* histone spacers.

*SAR.* Laemmli and coworkers described the existence of a scaffold attachment region (SAR) in the H1 - H3 spacer of the *D. melanogaster* histone repeat unit (Mirkovitch *et al.* 1984; Gasser and Laemmli 1986a,b).

It is suggested that SARs may border functional units in the chromatin and in that way are involved in the expression of genes (for review see Gasser and Laemmli 1987). Since the basic organization of the histone genes in *D. hydei* is comparable to that in *D. melanogaster* we analysed the *D. hydei* histone gene cluster for the existence of a SAR with the criteria given by Gasser and Laemmli (1986a and b). These criteria are: (1) an enrichment of sequences homologous to the topoisomerase II cleavage site consensus sequence (GTNA/TAC/TATTNATNNG) (Sander and Hsieh 1985) with the 6 bp surrounding the cleavage site conserved (underlined) and an overall homology of more than 70% and (2) the occurrence of A boxes (AATAAAT/CAAA) and T boxes (TTA/TTT/ATTT/ATT). According to the sequences of the A and T boxes shown by Gasser and Laemmli (1986a) we screened the sequence of the *D. hydei* histone gene cluster with the consensus sequence for these two elements allowing two mismatches. The same analyses were done for the sequence of the *D. melanogaster* histone repeat unit as published by Matsuo and Yamazaki (1989). The results are shown in Table 1. In *D. melanogaster* the H1 - H3 spacer shows a clear enrichment of sequences homologous to the topoisomerase II cleavage site and both A and T boxes occur. In *D. hydei*, however, the enrichment in topoisomerase II cleavage sites is less prominent. In addition, both the H2B - H1 and to a lesser extent the H4 - H2A spacer regions show more topoisomerase II cleavage site homologies per 100 bp than the H1 - H3 spacer region and in addition contain A and T boxes. This picture of topoisomerase II cleavage site enrichment did not change when we only looked at the region of nucleotides 1280 - 1990 which is comparable to the SAR in the *D. melanogaster* H1 - H3 spacer, according to its position. In *D. hydei* 1.4 topoisomerase II cleavage site homologies per 100 bp are found in that limited region. Hence, in contrast to the situation in *D. melanogaster*, the H2B - H1 spacer in *D. hydei*, would be the most likely candidate for having a SAR. This result is unexpected from the point of view that the basic organization of the histone gene cluster of both *Drosophila* species is the same. However, we cannot exclude that the detection of potential SAR sites in *D. hydei* is not possible with the criteria we used. Consensus sequences for topoisomerase II cleavage sites, A boxes and T boxes might be slightly different in *D. hydei* in comparison to those in *D. melanogaster*.

| | TopoisomeraseII sites/100 bp | | A boxes /100 bp | | T boxes /100 bp | |
|---|---|---|---|---|---|---|
| | Dh | Dm | Dh | Dm | Dh | Dm |
| H1 - H3 | 1.2 | 0.9* | 2.2 | 2.6 | 9.3 | 11.4 |
| H3 - H4 | 0.0 | 0.0 | 1.3 | 0.3 | 3.2 | 1.0 |
| H4 - H2A | 1.3 | 0.0 | 0.6 | 0.8 | 3.7 | 4.0 |
| H2A - H2B | 0.8 | 0.4 | 0.4 | 0.4 | 8.1 | 2.6 |
| H2B - H1 | 1.8 | 0.7 | 1.0 | 2.2 | 6.6 | 7.0 |
| H1 | 0.1 | 0.1 | 0.4 | 0.4 | 1.7 | 1.8 |
| H3 | 0.0 | 0.0 | 0.5 | 0.0 | 0.7 | 0.0 |
| H4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| H2A | 0.0 | 0.3 | 0.0 | 0.0 | 0.5 | 0.0 |
| H2B | 0.8 | 0.3 | 0.3 | 0.0 | 0.5 | 1.1 |

**Table 1.**. Occurrence of topoisomerase II sites, A boxes and T boxes in both orientations in the protein and non protein coding regions of the histone repeat unit of *D. hydei* (Dh) and *D. melanogaster* (Dm) as published by Matsuo and Yamazaki (1989). The number of sites are given per 100 bp. *, In contrast to Gasser and Laemmli (1986b) we find only 10 topoisomerase II sites in the HinfI - EcoRI fragment (including the SAR region) by using the criteria defined by the authors. They give the number of 18.

*Comparison of the D. hydei and D. melanogaster histone repeat unit.*

As described before, the arrangement of the histone genes within the repeat unit is closely similar in *D. hydei* and *D. melanogaster*. The protein coding regions of the core histone genes of both species are of the same length. The H1 protein coding region of *D. melanogaster*, however, codes for a protein which is 7 amino acids longer than that of *D. hydei* (see below). Otherwise the difference in length between the repeat units of both species is due to length differences in the spacer regions. The length differences of the spacers between the divergently transcribed H2A - H2B genes (247 and 226, respectively) and H3 - H4 genes (316 and 296, respectively) is smaller than that of the spacers between H1 - H2B (510 and 406, respectively) and H2A - H4 genes (609 and

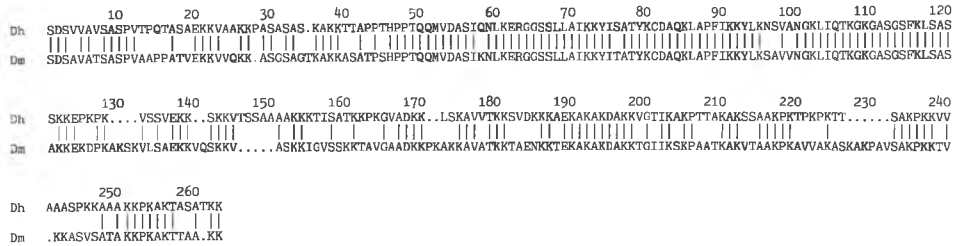



**Fig. 5. Alignment of the amino acid sequence of H1 of *D. hydei* and *D. melanogaster*. Gaps are indicated by *dots*, identical residues are marked by *dashes*.**

474, respectively) The length differences of the spacers between the H1 - H3 genes of both species (1253 and 1155, respectively) is comparable to that of the divergently transcribed genes when we exclude the tRNA derived insertion in *D. melanogaster*. This might be correlated to the existence of a SAR in the H1 - H3 spacer in *D. melanogaster* (see above). The high degree of length conservation of the spacers in the H2A - H2B and H3 - H4 spacers is barely reflected in sequence conservation.

*Protein coding sequences*. We compared the amino acid sequence of the histones as it can be deduced from the sequence of clone pDhH6/7 of *D. hydei* (Fig. 3) with those given by Matsuo and Yamazaki (1989) for *D. melanogaster*. The amino acid sequences of the core histones are identical (H4 and H2A) or nearly identical (H3 and H2B). In H3 there is one conservative amino acid substitution in position 117 which is a valine in *D. hydei* and an isoleucine in *D. melanogaster*. In H2B a nonconservative amino acid substitution from asparagine in *D. hydei* by threonine in *D. melanogaster* in the N-terminal region of the protein at position 22. The N-terminal region is the most variable region of H2B (Isenberg 1979).

For all organisms H1 is the least conserved of all histones (Elgin and Weintraub 1975). The N and C terminal part generally show a high degree of sequence variation in comparison to the globular part which includes a central stretch of about 80 amino acids (Isenberg 1979; Von Holt et al. 1979; Cole 1984). An alignment of the H1 amino acid sequence of both species was made (Fig. 5). The amino acids 44 - 128 form a central highly conserved region of 85 amino acids. The homology (amino acid level) in that region is 89% while in the N (43 amino acids) and C terminal part (127 and 120 amino acids in *D. hydei* and *D. melanogaster*, respectively) it is 63% and 61%, respectively. The alignment shows that the length difference of 7 amino acids between H1 of *D. hydei* and *D. melanogaster* is located in the C terminal region of the protein.

*Non protein-coding regions*. We analysed the conservation of the non protein coding-regions of the D. *hydei* and *D. melanogaster* (Matsuo and Yamazaki 1989) repeats by alignment. In general the homology between corresponding non coding regions is very limited. We found that the regions upstream (= total H2A - H2B and H3 - H4 spacer; 200 bp for the H1 gene) and downstream (150 bp) of the protein coding regions are slightly more conserved than the remaining spacer sequences (Table 2). The 5′ regions show short perfectly

| | % SSS | %SSS/Myr |
|---|---|---|
| H3 | 125 | 1.0-2.1 |
| H4 | 90 | 0.8-1.5 |
| H2A | 132 | 1.1-2.2 |
| H2B | 135 | 1.1-2.3 |

**Table 2.** The percentage of silent site substitutions between the histone genes of *D. hydei* and *D. melanogaster* were calculated using the program diverge of the GCG software (method Perler *et al.* 1980). Because of restrictions in the program it was not possible to calculate a percentage of silent site substitutions corrected for multiple hits for the highly diverged H1 genes. The time of divergence between *D. hydei* and *D. melanogaster* is estimated to range between 30 and 60 million years (Beverley and Wilson 1984; Throckmorton 1975). The two values given in column 2 represent the percentage silent site substitution per million years using the two extreme values of the divergence time. Myr, million years; *sss*, silent site substitutions

matching regions of 5 - 10 bp (a 16 bp region for H1), in addition to the TATA boxes and the presumptive cap sites. These sequence elements are marked in the alignment (fFig. 4A). For all four core histone genes two conserved sequence elements (one larger block for H4) are found between 30 and 70 bp upstream of the presumptive cap site (Fig. 4B and C). Because the core histone genes are divergently transcribed the promoter regions of H2A and H2B genes might be overlapping (*cf.* chicken H2A - H2B spacer region (Sturm *et al.* 1988). So we cannot assign specific elements to specific genes. The same situation exists for the H3 and H4 genes. In the H3 upstream region in the *D. hydei* repeat unit part of the homology between *D. melanogaster* and *D. hydei* is repeated immediately downstream of the homologous sequence block.

The H1 upstream regions are difficult to align. A conserved sequence element of 16 bp (TCCTCCTCGATTCTCA) (pos. 1032) is found which has a different location upstream of the presumptive cap site of *D. hydei* H1 in comparison to *D. melanogaster*. We marked conserved sequence elements in an alignment based on the 16 bp conserved region and the cap sites. Whether or not the marked sequence elements are functional has to be determined experimentally. Sequences of further *Drosophila* species which could give additional information are unknown.

*Silent site substitution rate.* Histones, with the exception of H1, are highly conserved proteins (Elgin and Weintraub 1975) and amino acid substitution rates can only be calculated over relatively long periods of divergence. However, the phylogenetic distance between *D. hydei* and *D. melanogaster* results in a significant amount of silent site substitutions at the DNA level. Busslinger *et al.* (1982) showed that the highly repeated sea urchin histone genes, coding for H3 and H4 necessary in early embryogenesis, evolved at the silent sites with a constant rate of 0.5-0.6 % base changes-Myr (million years) (method Perler *et al.* 1980). We used the same method (GCG Sequence Analysis Software Package) to calculate the silent site substitution rate for the *Drosophila* histone genes (table 3). The substitution rate for H4 of *Drosophila* is about 1.3-3 times higher than for sea urchin. The rate for *Drosophila* H3 is 1.7-4.2 times higher than that for sea urchin H3. The values for H2A and H2B are comparable to those for H3.

Our results show that the silent site substitution rate for histone genes in *Drosophila* is generally higher than in sea urchins and thus that histone genes in different organisms do not mutate with a constant rate at silent sites. This is in agreement with data, showing that the rates of silent site substitutions vary among groups of animals and among genes (Britten 1986; Li *et al.* 1987; Sharp and Li 1989).

The differences in the rates of silent site substitution might result from differences in the generation times, which differ largely between *Drosophila* and sea urchin, and/or selection pressure on codon usage as discussed by Britten (1986) and Li *et al.* (1987).

*Conclusions*

The genomic organization of the histone genes of *D. hydei* resembles closely that of *D. melanogaster*. The DNA sequence similarity in the 5' and 3' regions of the individual histone genes between *D. hydei* and *D. melanogaster* indicate that the regulation of transcription of the histone genes in the genus *Drosophila*, and possibly in insects in general, involves regulatory elements different from other higher eukaryotes.

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Chapter IV

# Transcription of histone genes in *Drosophila hydei*: Indications for the presence of histone variants

HANNIE KREMER AND WOLFGANG HENNIG

**Abtract.** To analyse histone gene expression in spermatogenesis, transcription of histone genes was studied in testes of males of *Drosophila hydei*. For comparison male carcass RNA (*i.e.* RNA from males without testes) was analyzed. DNA sequences of the presumptive cell-cycle regulated histone genes of *D. hydei* were used as a probe. The predominant histone messengers in both testis and carcass RNA are those with the length expected for the presumptive cell-cycle regulated histones. In addition to these, histone messengers where detected which might code for replacement variants. Some of these variant messengers were specific for testis, others for somatic tissues. The putative (testis-specific) histone replacement variants in testes are candidates for substitution of the presumptive cell-cycle regulated histones in male germ cells. A second and third type of variant histone messengers are those which only differ in length or both in length and occurrence in the poly(A)$^+$ fraction from the presumptive cell-cycle regulated ones, but not in sequence.

## Introduction

In many organisms a complex process of chromatin reorganization occurs during spermatogenesis. It is related to the packaging of the chromatin which occurs during and after meiosis.

Chromatin reorganization is usually accompanied by a substitution of the somatic histones by other proteins, mainly testis-specific histone variants and eventually by protamines or other proteins more basic than histones (for review see Bloch 1969; Poccia 1986; Hecht 1989; Risley 1989).

Only very restricted information on chromatin reorganization is available for *Drosophila* spermatogenesis. Cytochemical experiments implicated that in elongated spermatid nuclei of *D. melanogaster* arginine-rich proteins are substituted for lysine-rich nuclear proteins (Das *et al.* 1964; Hauschteck-Jungen and Hartl 1982). Whether this substitution is a last step of a series of consecutive events replacing somatic histones by variants is unclear. In *D. hydei* we demonstrated immunocytochemically that histone H2A is present in the spermatid nucleus until late elongation. However, histone H1, which was found in spermatogonia and in early primary spermatocytes, is immunocytochemically not detectable in the chromatin of all subsequent stages of spermatogenesis (Kremer *et al.* 1986). These observations cannot prove whether the histone H2A detected in spermatid nuclei is the ubiquitous somatic histone and whether somatic histone H1 is entirely removed from the chromatin at the onset of the meiotic prophase. The reaction of H2A antiserum might occur with a conserved antigenic determinant of a variant H2A histone. Histone H1, on the other hand, might only be present in small amounts escaping detection by immunofluorescence. It may be masked and therefore not be detectable, or it may be substituted for by a H1 histone variant not recognized by the antiserum against somatic histone H1.

The immunocytological observations (Kremer *et al.* 1986) induced the present analysis of the transcription of the histone genes in testes of *D. hydei*. We used separate probes specific for each of the histone genes in a Northern blot analysis of testis RNA and, for comparison, of carcass RNA (*i.e.* RNA

from males without testes). In addition to the somatic presumptive cell-cycle regulated histone mRNAs (Kremer and Hennig 1990) we found other RNA species with sequence similarity to histone genes. Some of these RNA species are specifically expressed in testes while others are expressed in somatic tissues as well. Our observations are discussed in the context of chromatin rearrangements during spermatogenesis of *D. hydei* (see also chapter V).

## Materials and methods

*Drosophila strain.* The wildtype strain of *D. hydei* was from our laboratory collection.

*Isolation of RNA.* RNA from testes and carcass (*i.e.* males without testes) of *D. hydei* wild-type flies was isolated as described by Brand and Hennig (1989). For separation of the poly(A)$^+$ and poly(A)$^-$ RNA the mRNA purification kit of Pharmacia was used according to the Pharmacia protocols.

*RNA blot hybridization.* Glyoxal/DMSO denaturated RNA was separated on horizontal 2% agarose gels, blotted onto Hybond N (Amersham) and hybridized as described by Thomas (1980). Hybridization temperature was 60°C, washing temperature 50°C. Nonstringent washing was done with 0.3 M $Na_2HPO_4$, pH 7.2, 1% SDS, stringent washing with 0.02 M $Na_2HPO_4$, pH 7.2. RNA ladders of BRL were used as size-markers.

*Cloning of gene-specific DNA fragments.* To obtain a specific probe for each of the histone genes, we subcloned fragments of the histone repeat unit of *D. hydei*, pDhH6/7 (Kremer and Hennig 1990), into M13 mp18/19 (Messing and Vieira 1982; Yanish-Perron *et al.* 1985). The location of the gene-specific probes within this repeat unit is shown in table 1 and figure 1. Cloning of the fragments and isolation of single stranded templates was performed according to the Amersham protocols (Amersham 1984). The nucleotide sequences of the cloned fragments were verified by the dideoxy chain termination method (Sanger *et al.* 1977).

*Isolation and labelling of gene-specific probes.* DNA of the gene-specific fragments was obtained by amplification of single-stranded templates with the polymerase chain reaction (PCR). As primers the M13 sequencing (-20) and the reversed sequencing primers (Pharmacia) were used. The PCR-reaction mixture of 100 µl contained 0.5-1.0 µg of single stranded template, 0.2 mM of each of the dNTPs, 0.5 µg of each of the primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine and 2.5 U *Taq* polymerase (Perkin-Elmer-Cetus). The first step of denaturation was at 94°C for 5 min. Then *Taq* polymerase was added and the mixture was overlaid with 50 µl of mineral oil (Merck). 25 cycles of a step program (94°C, 1 min; 55°C, 2 min; 72°C, 2.5 min with an extension of 1 s at every cycle) followed by a 15 min final extension were performed for amplification. The reaction mixture was extracted with 100 µl of chloroform according to the protocol of Perkin-Elmer-Cetus in order to remove the mineral oil before precipitation of the products

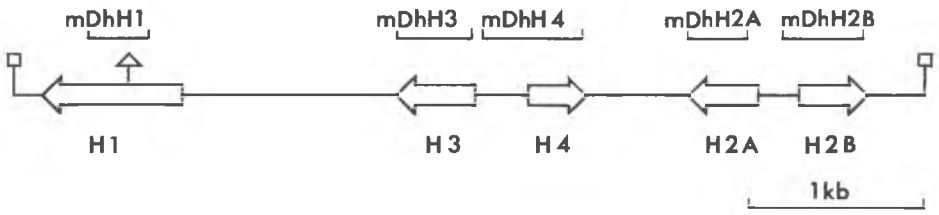


**Fig. 1.** Schematic representation of the location of the gene-specific probes within the histone repeat unit pDhH6/7 (Kremer and Hennig 1990). The *arrows* mark the direction of transcription of the genes. Bam H1, ⊓ ; Eco RI, ⊓

with ethanol. The flanking sequences of the multiple cloning site of the M13 vector were removed by restriction digestion. Inserts and M13 fragments were separated on horizontal 2% agarose gels and subsequently the inserts were isolated from the low melting agarose. Labelling of the inserts was done by nick-translation according to the protocols of Maniatis *et al.* (1982). For direct labelling of the H1-specific probe with PCR, the reaction was performed as described above with the following modifications: 0.25 µg of each of the primers were used, dCTP was used in a concentration of 25 µM and 100 µCi of α-[$^{32}$P]dCTP (3000 Ci/mmol, Amersham) were added.

## Results

Insert DNA of the clone pDhH6/7 containing one copy of each of the presumptive cell-cycle regulated histone genes H1, H2A, H2B, H3 and H4 (Kremer and Hennig 1990) was used as a hybridization probe on Northern blots of total testis RNA and carcass RNA (Fig. 2). The main hybridization signals in both lanes correspond to RNAs of about 900 nt, which corresponds to the length expected for histone H1 mRNA, and to RNA of about 400 to 500 nt, which corresponds to the length expected for the messengers of the core histone genes (Kremer and Hennig 1990, *cf.* Table 2). Therefore, these major histone transcripts are referred to as the presumptive cell-cycle regulated mRNAs.

In contrast to previous studies on *D. melanogaster* histone gene transcripts (Burkhardt and

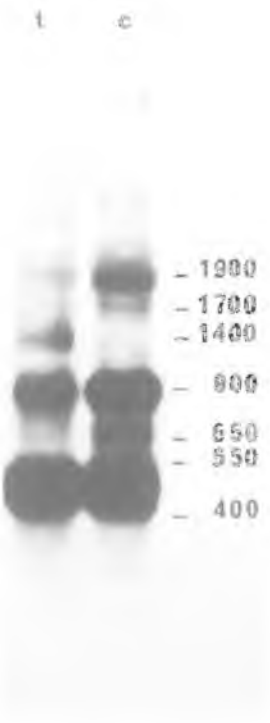


**Fig. 2.** Northern blot hybridization with nick-translated pDhH6/7 insert DNA as a probe on 20 µg of total RNA from testis (t) and carcass (c). After hybridization the blot was washed using nonstringent conditions (0.3 M $Na_2HPO_4$, 50 °C). BRL size markers (1.77 - 0.16 kb) were used for length determination. The lengths of the transcripts are given in nucleotides.

**Table 1.** Location of the histone gene-specific fragments within the repeat unit pDhH6/7 (Kremer and Hennig 1990).

| probe | gene specificity | location in pDh6/7 (nt) |
|---|---|---|
| mDhH1 | H1 | 428 - 761 |
| mDhH2A | H2A | 3799 - 4151 |
| mDhH2B | H2B | 4357 - 4804 |
| mDhH3 | H3 | 2160 - 2581 * |
| mDhH4 | H4 | 2648 - 3201 |

The location of the fragments is given in nucleotides according to the numbering in Kremer and Hennig (1990). The fragment marked with a * is inserted twice in the M13 vector.

Birnstiel 1978; Anderson and Lengyel 1980, 1984; Farrell-Towt and Sanders 1984) we find RNA species in addition to the presumptive cell-cycle regulated histone mRNAs. Since the major part of these hybridization signals decrease using stringent washing conditions (data not shown), we conclude that they indicate the existence of mRNA of histone variants with divergent nucleotide sequences.

*Gene-specific transcripts*

For further detailed analyses of these additional transcripts, probes specific for each of the histone genes were obtained by subcloning fragments of the histone repeat unit of pDhH6/7 into M13 mp18/19 (Fig. 1, Table 1). To discriminate between possible sequence variants and length variants simply produced by the addition of a poly(A) tail, two stringencies (0.3 M and 0.02 M $Na_2HPO_4$) were used in the washing procedure after hybridization of the gene-specific probes to blots of total testis and carcass RNA (data not shown).

With each of the gene-specific probes we found hybridization to transcripts larger than the cell-cycle regulated ones. Some of these signals are weak. In contrast to the messengers with a length corresponding to that deduced from the loading sequences of pDhH6/7, the majority of the additional histone transcripts is found in the poly(A) fraction of the RNA and does not seem to be identical in sequence to that of the probes (Fig. 3, Table 2).

**Fig. 3 a,b.** Northern blot hybridizations of the gene-specific probes, described in figure 1 and table 1, on testis and carcass RNA, using nonstringent washing conditions (0.3 M $Na_2HPO_4$, 50 °C). a, Hybridization on the poly(A)$^+$ and poly(A)$^-$ fractions of testis and carcass RNA. About 1 µg of poly(A)$^+$ RNA and about 20 µg of poly(A)$^-$ RNA were loaded. b, Hybridization of the H1-specific probe on about 20 µg of total testis and carcass RNA. The H1-specific fragment was directly labelled in the PCR reaction. Size markers were from BRL (1.77 - 0.16 kb). The lengths of the transcripts are given in nucleotides. t, testis; c, carcass; A$^+$, poly(A)$^+$ fraction; A$^-$, poly(A)$^-$ fraction.

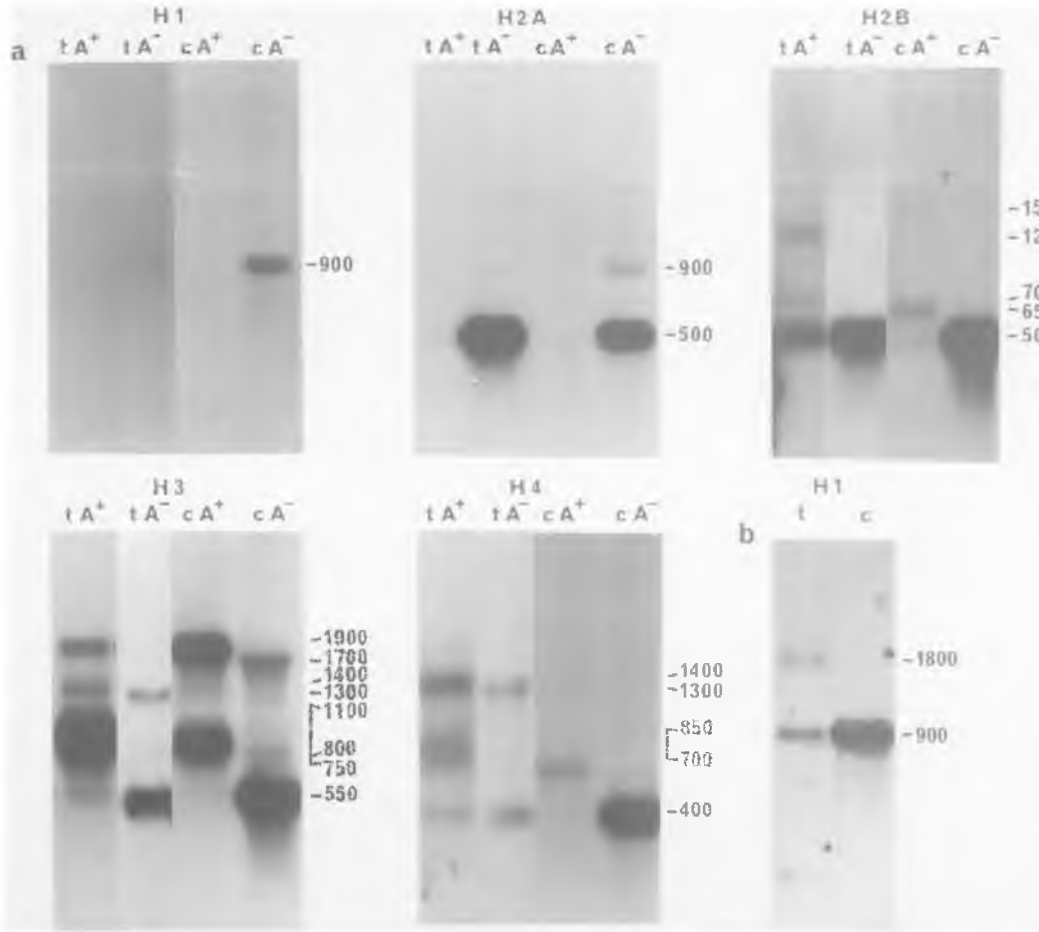
a
H1
tA+ tA− cA+ cA−
-900
H2A
tA+ tA− cA+ cA−
-900
-500
H2B
tA+ tA− cA+ cA−
H3
tA+ tA− cA+ cA−
-1900
-1700
-1400
-1300
-1100
-800
-750
-550
H4
tA+ tA− cA+ cA−
-1400
-1300
-850
-700
-400
b
H1
t c
-1800
-900

**Table 2.** Schematic representation of the results presented in figure 3.

| Gene | Testis | | Carcass | | pDhH6/7 |
|---|---|---|---|---|---|
| | $A^+$ | $A^-$ | $A^+$ | $A^-$ | |
| H1 | 1800● | 900° | | 900° | 880 |
| H2A | | 900°<br>500° | | 900°<br>500° | 480 |
| H2B | 1200*<br>700● | 500° | 1550*<br>650● | 500° | 470 |
| H3 | 1900*<br>1400°<br>[1100<br>750]* | 1300°<br>550° | 1900*<br>[1100<br>800]* | 1700°<br>550° | 530 |
| H4 | 1400°<br>[850<br>700]* | 1300°<br>400° | 700* | 400° | 425 |

The length of the nucleotide sequence of RNA fractions in "Testis" and "Carcass" is calculated from their electrophoretic mobilities. BRL size markers (1.77 - 0.16 kb) were used. The length of mRNAs in nucleotides as deduced from the clone pDhH6/7 Kremer and Hennig 1990) is shown in the column "pDhH6/7". *, limited sequence similarity to the probe; o, high sequence similarity to the probe; ●, no data about decrease of signal intensity after stringent washing; [ ] upper and lower limit of diffuse band.

*Histone H1.* With the H1-specific probe, mDhH1, we only found one additional RNA of a length of ~1800 nt in testes (Fig. 3b). In further experiments we have to determine whether this RNA species is polyadenylated. The signal of the presumptive cell-cycle regulated H1 RNA with a length of ~900 nt is considerably weaker in testis than in carcass RNA.

*Histone H2A.* Hybridization with the H2A-specific probe, mDhH2A, shows in both the testis and carcass poly(A)$^-$ RNA fractions an RNA species of ~900 nt in length in addition to the presumptive cell-cycle regulated mRNA with a length

of ~500 nt (Fig. 3a). Sequence divergence from the cell-cycle regulated mRNA is low or absent since stringent washing showed no decrease in the hybridization signal of the ~900 nt RNA. Consequently, we have no evidence for H2A sequence variants. Several alternative explanations for the length difference of the ~ 900 nt RNA species with the presumptive cell-cycle regulated histone messengers will be discussed later (see discussion).

*Histone H2B.* The H2B-specific hybridization probe, mDhH2B, detects two testis-specific poly(A)$^+$ (~700 nt and ~1200 nt) and two carcass-specific poly(A)$^+$ RNA species (~650 nt and ~1550 nt) in addition to the presumptive cell-cycle regulated poly(A)$^-$ H2B RNA (~500 nt) (Fig 3a). Both the ~1200 nt and the ~1550 nt poly (A)$^+$ RNAs are divergent in their sequence from the H2B probe since stringent washing did clearly decrease the hybridization signal. We cannot distinguish yet whether also the ~650 nt and ~700 nt RNA species are candidates for messengers coding for histone sequence variants. The hybridization signals of these RNA species were too weak in the experiments where both stringent and nonstringent washing conditions were compared, because total testis and carcass RNA were used in these experiments. A relatively higher enrichment of the ~650 nt and ~700 nt transcripts in comparison to the ~1200 nt and ~1500 nt RNA species must have occurred during separation of the poly(A)$^+$ and poly(A)$^-$ RNA fractions since the relative strength of the hybridization signals of the RNA species changed (data not shown).

*Histone H3.* With the H3-specific probe, mDhH3, a complex hybridization pattern appeared (Fig 3a). In the testis poly(A)$^+$ RNA fraction we found a diffuse signal in the region corresponding to RNA species ~750-1100 nt in length and two discrete signals reflecting RNA species of ~1400 and ~1900 nt. A weak exposure of the Northern blot showed that the diffuse signal is not caused by a distinct double-band (data not shown). Both the 750-1100 nt and the 1900 nt RNAs show a strong decrease in the signal after stringent washing which indicates that these RNA species are candidates for coding for an H3 sequence variant in testis.

Both the poly(A)$^+$ 1400 nt and the poly(A)$^-$ 1300 nt RNA species from testis show a high degree of sequence similarity to the H3-specific probe since stringency of washing did not affect the corresponding signals on the Northern blot. It is remarkable that both signals also appear with the H4-specific probe, mDhH4 (Fig 3a). This probe includes 250 bp 5' upstream of the H4 gene; *i.e.* it contains the H3-H4 intergenic region up to the presumptive TATA box of the H3 gene (Fig. 1).

Hybridization of the H3-specific probe with carcass poly(A)$^+$ RNA detected discrete transcripts of ~1900 nt and heterogeneous RNAs of ~800-1100 nt in length. The clear decrease of the signals under stringent washing conditions indicates the presence of sequence variants of histone H3 in carcass. In the poly(A)$^-$ fraction we found an RNA species of ~1700 nt in addition to the presumptive cell-cycle regulated one (~500 nt). The sequence similarity between the probe and the RNA species of ~1700 nt must be high because of the lack of a clear decrease of the hybridization signal after stringent washing.

*Histone H4.* With the H4-specific probe, mDhH4, we found a diffuse hybridization signal with poly(A)$^+$ testis RNAs of ~700-850 nt. These RNAs cannot be

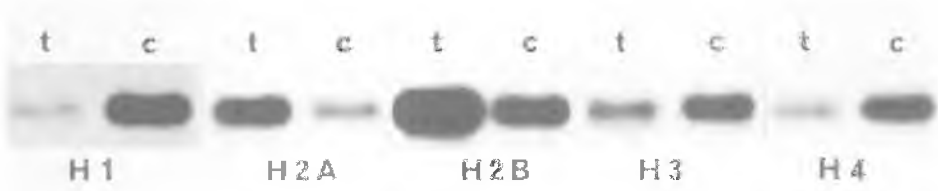


**Fig. 4.** Abundance of histone mRNAs. Northern blot hybridization with the gene-specific fragments, described in figure 1 and table 1, as a probe. The region of the presumptive cell-cycle regulated messengers is shown. About 20 μg of total testis and carcass RNA were loaded in each lane. The presence of equal amounts of RNA per lane was confirmed through hybrdization with a DNA probe derived from the ribosomal RNA genes. Stringent washing conditions (0.02 M $Na_2HPO_4$, 50 °C) were used. t, testis; c, carcass.

detected in carcass RNA. The hybridization of the poly(A)$^-$ RNA of ~1300 nt and the poly(A)$^+$ RNA of ~1400 nt in testis RNA was already described above.

The carcass poly(A)$^+$ fraction showed a carcass-specific RNA species of ~700 nt after hybridization with the H4-specific probe.

Interpretation of signals after washing under stringent conditions was difficult since only a slight decrease in signal intensity was found in the length range of 700-850 nt in both testis and carcass RNA. Therefore probably some sequence variation may exist between the H4-specific probe and these messengers.

*Relative amounts of the presumptive cell-cycle regulated messengers.* Additional information on the expression of histone genes is obtained by comparing the intensity of the hybridization signals of the presumptive cell-cycle regulated poly(A)$^-$ histon mRNAs in testis versus carcass RNA (Fig. 4).

While the H2A - and H2B - specific probes hybridize stronger with testis RNA than with carcass RNA, for H3 and H4 the hybridization on carcass RNA is more intense. Also with the H1-specific probe the hybridization signal for the presumptive cell-cycle regulated H1 RNA was considerably weaker for testis RNA than for carcass RNA.

## Discussion

In RNA extracted from embryos and cultured cells of *D. melanogaster* five different histone mRNAs were described (Anderson and Lengyel 1980, 1984) which are not polyadenylated. In length they correspond to the sizes expected from the genes coding for the presumptive cell-cycle regulated histones (Matsuo and Yamazaki 1989). Furthermore, a poly(A)-tailed mRNA coding for the H2AvD variant was described (van Daal *et al.* 1988).

In our analysis of the transcripts of histone genes in adult males of *D. hydei*, the RNA patterns are more complex (see Table 2). The RNA species responsible for the major hybridization signals had a length corresponding to those which could be deduced from the genes coding for the presumptive cell-cycle regulated histones (Kremer and Hennig 1990). As expected (Birnstiel

*et al.* 1985) these signals were found in the poly(A)$^-$ RNA fractions. Each of the probes specific for one of the histone genes disclosed additional RNA species on Northern blots of testis RNA or of male carcass RNA differing in one or more of the following aspects from those coding for the presumptive cell-cycle regulated histones: length, sequence and presence in the poly(A)$^+$ fraction. Both testis- and carcass-specific histone RNA species were found.

*Types of histone RNA variation*

The additional histone RNAs described above can be separated into three types which will be discussed separately.

*(1) Length variants in poly(A)$^-$ RNA.* Hybridization with the probes specific for H2A, H3 and H4 resulted in signals on RNA fractions which showed length differences (H2A: a 900 nt RNA species in testis and carcass RNA; H3: a 1300 nt testis RNA, a 1700 nt carcass RNA; H4: a 1300 nt testis RNA; see Table 2). Stringency of washing did not affect the hybridization signals on the Northern blots. Therefore, the sequence similarity to the radioactive probes is high.

The length differences could have various reasons. There might be several types of rearrangements of some genes located within or outside the histone gene cluster. A known example is the insertion of a transposable element within histone repeats of *D. melanogaster* although these insertion are in nontranscribed regions (*cf.* Ikenaga *et al.* 1982; Matsuo and Yamazaki 1989). Another explanation for longer transcripts might be an origin from one (or several) gene(s) within or outside the histone gene cluster with deviating 5' and/or 3' sequences causing tissue-specific transcription (*cf* Childs *et al.* 1981). For *D. hydei* further data are required to prove whether one of these or another explanation is valid. *In situ* hybridization on polytene chromosomes using $^3$H-labelled gene-specific fragments as a probe, gave only for histone H3 signals at a location different from that of the histone gene cluster (see chapter V).

*(2) Length variants in the poly (A)$^+$ RNA fraction.* With both the H3- and H4-specific probe we detected a polyadenylated RNA species of a length of ~1400 nt in testis RNA. Since the hybridization signal is not affected by changing the stringency of the washing conditions, these transcripts must have a high degree of sequence similarity with the sequence of the probes. Both properties, length difference and high sequence similarity to the probes, agree with the properties of similar length variants (~1900 nt) found in the poly(A)$^-$ RNA of testes. One possibility is, therefore, that the lenght difference is due to presence or absence of polyadenylation. Such differences were observed for a presumptively cell-cycle regulated H1 gene in several mouse and chicken tissues, and for an H2B gene in mouse spermatids (Challoner *et al.* 1989; Cheng *et al.* 1989; Kirsh *et al.* 1989). Moreover, the identical properties of the transcripts, namely hybridization with both H3 and H4 probes as well as their occurrence in both poly(A)$^-$ and poly(A)$^+$ RNA suggests that they may represent read-through transcripts of the H3-H4 region (see Fig. 1). The length is compatible with the expected size. In contradiction to such a possibility is

that H3 and H4 are transcribed in an opposite direction in the known repeat unit (Kremer and Hennig 1990). We have, however, no evidence, that this holds true for all repeat units. Otherwise, it can also not be excluded that the transcripts partly consists of the antistrand. Experiments with defined single-stranded probes so far have not been carried out and it has not been investigated whether the entire H4 transcribed region is present in both transcripts or only the 5' upstream region of an H4 gene since the available H4-specific probe, mDhH4, contains 250 bp of the H4-upstream region in addition to the H4 protein coding sequences (see Fig. 1). From the available data we cannot even decide whether the same transcripts hybridize with both probes or whether the 1400 nt RNA fraction is a mixture of different RNA species. It seems less likely, however, that in both the poly(A)$^+$ and poly(A)$^-$ fractions different transcripts of an H3 and an H4 gene coexist which simply by chance have the same length.

The ~1300 and ~1400 nt transcripts are testis-specific as far as Northern blots allow to conclude. Cloning of the corresponding cDNAs and genomic DNA fragments are necessary to elucidate details on these transcripts.

*(3) Sequence variants* In the prior sections we have discussed two types of RNA species, those of lengths different from the somatic histone mRNAs and those appearing in the poly(A)$^+$ fraction. A third type of RNA species is detected which differs in three aspects from its presumptive cell-cycle regulated counterpart. RNA species belonging to this type (a) are longer than the latter, (b) they differ in their nucleotide sequence since stringent washing conditions decreased the strength of the hybrization signals and (c) they occur in the poly(A)$^+$ fraction. This holds true for the RNAs of ~1200 and ~1550 nt detected with the H2B-specific probe and for the ~700-850 nt and ~700 nt RNA species detected with the H4-specific probe in testis and carcass RNA respectively (Fig. 3, Table 2). These characteristics also apply to the H3-similar RNA species of ~1900 nt in testis and carcass RNA and the ~750-1100 nt and ~800-1100 nt RNA species in testis and carcass respectively (Fig. 3, Table 2).

One might argue that the sequence divergence of these RNA species may be only due to silent nucleotide substitutions in different genes of the gene cluster or, for histone H4, in the 5' region of the gene(s). However, the enrichment in the poly(A)$^+$ fraction as well as a considerable increase in length in comparison to the cell-cycle regulated counterparts are characteristics of histone mRNAs coding for histone replacement variants (Brush *et al.* 1985, for review see Schümperli 1986). In addition diffuse hybridization signals have been described as characteristically observed for histone variant messengers in chicken and mouse (Engel *et al.* 1982; Hraba-Renevey and Kress 1989). A heterogeneity in the length of the presumptive poly(A)-tail of these RNAs might be the reason for this type of hybridization pattern.

Polyadenylated protamine and transition protein messengers show heterogeneity in the length of the poly(A) tracts in elongating mouse spermatids (for review see Hecht 1989). This heterogeneity is correlated with the onset of translation of the messengers after storage from early spermiogenesis on (Kleene *et al* 1984; Yelick *et al.* 1989). It is an interesting question whether a comparable situation holds true for the heterogeneous H3- and H4-like transcripts in *D. hydei*. In *D. hydei* spermatogenesis, transcription is not detectable after meiosis although protein synthesis occurs until late spermiogenesis (Hennig 1967).

The length heterogeneity detected in the above mentioned histone transcripts might reflect a regulatory mechanism for translation of stored transcripts comparable to that in mouse spermatids.

The only probe which did not give evidence for the existence of a replacement variant in *D. hydei* is the H2A-specific probe. In contrast, a cDNA and the corresponding gene coding for an H2A replacement variant (H2AvD) have been obtained from *D. melanogaster* (van Daal *et al.* 1988, 1990). The sequence similarity at the nucleotide level between the H2AvD gene and the presumptive cell-cycle regulated H2A gene is low. For this reason we cannot exclude the existence of an *D. hydei* gene corresponding to the H2AvD gene in *D. melanogaster*. The possibility of a low sequence similarity between the H2A-specific probe we used and a possible gene corresponding to the transcript of this presumptive variant gene in *D. hydei* might not allow us to detect this possible transcript. Therefore, a H2A variant may exist in *D. hydei* as well.

### *Differences in the abundance of core histone mRNAs*

Although one would expect a comparable amount of mRNAs coding for all core histones in both testis and carcass (*cf* Fig. 2 in Anderson and Lengyel, 1980), for each of the histone genes the intensity of the hybridization signal of the presumptive cell-cycle regulated poly(A)$^-$ mRNAs differed between testis and carcass. With the H2A- and H2B-specific probes, the hybridization signals in testis RNA are stronger than those in carcass RNA. For H3- and H4-specific probes a stronger signal of the presumptive cell-cycle regulated mRNA was found in the carcass RNA. A correlation may exist between these data and the fact that the abundance of RNA species different from the presumptive cell-cycle regulated ones seems to be much higher for H3 and H4 than for H2A and H2B.

The correlation between the intensity of hybridization signal of the presumptive cell-cycle regulated mRNAs in testis and the abundance of additional RNA species does not hold true for H1. However, we might not be able to detect additional H1 variant mRNAs because of potentially low sequence similarity with the probe. H1 genes are established as the histone genes most diverged in evolution (see Wells 1986 and Wells and McBride 1989).

### *Testis-specific histone variants ?*

We have discussed the presence of testis-specific and soma-specific transcripts which seem to differ only in length from the corresponding presumptive cell-cycle regulated messengers. They possibly contribute, in a cell-cycle independent way, to the fine regulation of the synthesis of the major type of histones, the cell-cycle regulated ones (*cf.* Cheng *et al.* 1989; Hraba-Renevey and Kress 1989).

After hybridization with the H2B- and the H4-specific probes we detected testis-specific RNAs of ~1200 nt and ~700-850 nt, respectively, differing in sequence from the corresponding cell-cycle regulated transcripts. The carcass-specific RNAs correspond to a length of ~1550 nt and ~700 nt, respectively. Several possible origins of these transcripts may be considered. The most direct

assumption is that testis- and carcass-specific RNA species code for different proteins. Alternatively, they might code for identical proteins but may be transcribed from different genes with a diverged nucleotide sequence. A third possibility is that the differing testis- and carcass-specific transcripts are transcribed form the same gene and code for identical proteins. The difference in transcript length might in this case be due to the tissue-specific use of different polyadenylation sites within one gene. Strong indications for the use of different polyadenylation sites of one gene have been described for the gene coding for the H3.3 replacement variant described to occur in mouse kidney cell cultures (Hraba-Renevey & Kress 1989). Further experiments are necessary to discriminate between the above mentioned possible origins of the testis- and carcass-specific transcripts in *D. hydei*.

Since the available data suggest the presence of testis-specific histone variants, it is an important question whether these variants are involved in histone transition during spermatogenesis. The low intensity of the hybridization signals from the testis-specific RNA species in comparison to those of the presumptive cell-cycle regulated ones argue against involvement in histone transition. However, the decrease of hybridization signals under stringent washing conditions, which is especially true for H2B, indicates a restricted sequence similarity between the hybridization probes and the messengers coding for histone variants. This can mask the real amount of messengers coding for histone variants. Furthermore, only part of the cells present in testis tissue are germ cells. With these arguments and the immunocytochemical data (Kremer *et al.* 1986) in mind, it is reasonable to assume that at least part of the testis-specific histone RNA species found in the poly(A)$^+$ fraction of testis RNA code for histone variants involved in histone transition. Only the analysis of histone cDNAs and the expression of the corresponding messengers can finally prove whether the detected poly(A)$^+$ and poly(A)$^-$ histone RNAs are functional messengers and whether at least part of them code for histones involved in histone transition during spermatogenesis. The first steps of such an analysis are described in chapter V.

**Acknowledgement** We like to thank Dr. Rein Brand for his support in the analysis of RNA.

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Chapter V

# Cloning of histone H3 cDNA fragments: A putative H3.3 histone variant in *Drosophila hydei*

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**Abstract.** We cloned cDNA fragments derived from both testis and carcass RNA. The amino acid sequences deduced from the uninterrupted open reading frames of the fragments indicate the presence of an equivalent of the H3.3 histone variant of vertebrates in *Drosophila hydei*. Multiple H3.3 transcript sizes can be detected in both testis and carcass poly(A)$^+$ RNA with different relative amounts. This indicates either a tissue-specific use of transcription signals or a tissue-specific splicing of the primary transcript. *In situ* hybridization on polytene chromosomes using a H3.3 cDNA fragment as a probe revealed signals at three different genomic locations including that of the presumptive cell-cycle regulated histone genes. This might indicate the existence of a multigene family coding for the putative H3.3 variant as is present in mammals.

## Introduction

The packaging of DNA into its highly condensed form in the nuclei of eukaryotic cells is mediated by histones in concert with non-histone chromosomal proteins.

Although histones are highly conserved during evolution, they show a certain degree of diversity. There exists amino acid sequence variants that can be divided into three major classes (for review see Schümperli 1986). The first class contains those histones which in expression are correlated with the replication of the cellular DNA. Since their synthesis is cell-cycle regulated we shall refer to them as "cell-cycle regulated histones". The histones of the second class, the "replacement variants", have a unique pattern of expression during development and cellular differentiation. They are expressed constitutively at a basal level throughout the cell-cycle. The third class includes all tissue-specific variants. An example is the erythrocyte-specific histone H5 (Tsai and Hnilica 1975). For each of the histones H2A, H2B and H3 a specific combination of representatives of all three classes can be found in different cell-types (for review see Stein *et al.* 1984).

Although the presumptive cell-cycle regulated histone genes of *Drosophila melanogaster* were among the first eukaryotic genes cloned (Karp and Hogness 1976), only recently a gene of this species coding for an H2A replacement variant was characterized (van Daal *et al.* 1988, 1990). Our transcript analysis of histone genes of *D. hydei* gave additional evidence for the existence of a variety of primary sequence variants of histones H2B, H3 and H4 in *Drosophila* (see chapter IV).

These observations are further substantiated by the data from cDNA cloning of three 240 bp fragments representing a specific region of variant histone H3 transcripts isolated from testis and carcass of *D. hydei*. The sequence of all three fragments represents an uninterrupted open reading frame. The conversion of the nucleotide sequence into an amino acid sequence reveals for one of the clones an amino acid sequence identical to the internal part of the H3.3 histone replacement variant of vertebrates. Northern blot analysis indicated that the gene coding for the presumptive H3.3 variant of *D. hydei* has the same structure as the vertebrate H3.3 gene. *In situ* hybridization demonstrats that the genomic sequences reside in two chromosomal positions different from that of the main histone gene cluster.

## Materials and methods

*Drosophila strain.* The wildtype strain of *D. hydei* was from our laboratory collection.

*Cloning of cDNAs from histone transcripts.* For cloning of cDNAs from histone transcripts we used a method modified from that described by Frohman *et al.* (1988) according to the following description.

*-cDNA(-)strand synthesis* (Fig. 1A,B). Total testis or carcass RNA (1-2 μg), isolated as described by Brand and Hennig (1989), was reverse transcribed in the following reaction mixture of 20 μl: 20 pmol of primer, 1x RT buffer (BRL), 10 U RNasin (Promega Biotec), 20 μCi α-[$^{32}$P]dCTP (Amersham), 200 U M-MLV reverse transcriptase (BRL) and each dNTP at a concentration of 0.5 mM. Prior to reverse transcription the RNA was dissolved in DEP-treated $H_2O$, heated at 65°C for 3 min and quenched on ice. As primers we used $(dT)_{17}$ -oligomer (Boehringer) or H3-primer I (see Fig. 1).

*-Tailing of the cDNA(-)strand* (Fig. 1C,D). Excess nucleotides and primers were separated from the products of reverse transcription using a Sephadex G-50 (fine) column (in a 2 ml serological pipette plugged with silane-treated glass wool), equilibrated with 0.05 x TE buffer (TE is 10 mM Tris-HCl, pH 7.5, 1 mM EDTA). During elution with 0.05 x TE buffer, about 50 one-drop fractions were collected. The fractions containing the first peak of radioactivity, together with the two preceeding fractions, were pooled and concentrated by centrifugation under reduced pressure. Subsequently the volume was adjusted to 23 μl. For tailing 1 μl of 6 mM dCTP, 6 μl of 5x tailing buffer (BRL) and 1 μl of 15 U/μl terminal deoxytransferase (BRL) were added. Before adding the enzyme, the reaction mixture was prewarmed at 37°C for 5 min. The tailing reaction was performed at 37°C for 8 min. After tailing, the reaction mixture was diluted to 500 μl with TE buffer.

*-Amplification* (Fig. 1E,F). For amplification of the 5' region of the histone transcripts a $(dG)_{12}$-adaptor primer (10 pmol) in combination with an adaptor primer (25 pmol), containing a Not I site, was used (Fig. 1E). 10 μl of the diluted tailing mixture was used for amplification in 50 μl of 1 x PCR buffer with each dNTP at a concentration of 0.2 mM, primers as mentioned above, and 2.5 U of *Taq* polymerase (Perking-Elmer-Cetus). The first cycle of amplification included a denaturation step of 5 min at 94°C, an annealing step of 2 min at 50°C and an elongation step of 30 min at 72°C. The *Taq* polymerase was added at the beginning of the elongation step. Subsequently the mixture was overlaid with 50 μl of mineral oil (Merck). For amplification 30 cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C (for the last step 1 sec extension per cycle) were performed. The last elongation step had a 15 min extension. For amplification of an internal region of the cDNA strand (Fig. 1F), the adaptor primer and the $(dG)_{12}$-adaptor primer were substituted by H3-primers II and III (25 pmol each). Mineral oil was removed by extraction with 100 μl of chloroform according to the protocol of Perkin-Elmer-Cetus and the PCR products were precipitated with ethanol.

*Analysis of the PCR products.* About one fifth of the PCR reaction mixture was electrophoresed on a horizontal 2% agarose gel and blotted onto Hybond N according to the Amersham protocols (Amersham 1985). The H3-oligonucleotide probe was labelled with γ-[$^{32}$P]dATP according to the protocol of Sambrook *et al.* (1989). Excess of γ-[$^{32}$P]dATP was removed by precipitation with ethanol.

About 10 pmol of probe were used per hybridization. (Pre-)hybridization was done according to BRL protocols. Prehybridization was performed in 2 x SSC (SSC is 0.15 M NaCl, 0.015M sodium citrate), 20 mM sodium phosphate (pH 7.0), 10 x Denhardt's solution (Denhardt 1966), 7% SDS for at least 2 h at 57°C. Hybridization was performed overnight at 57°C in fresh pre-hybridization solution adding 1/4 volume of 50% dextrane sulphate (Pharmacia). Washing was twice in 4 x SSC, 1% SDS at room temperature for 15 min and once at 57°C for 5-8 min.

*Cloning and sequencing of the PCR products.* PCR products were digested with Not I and separated on a horizontal gel of 3% low melting agarose (Nusieve). The DNA fragments were isolated and cloned into M13 mp18. The cloning protocols of Maniatis *et al.* (1982) were used. The nucleotide sequences were determined by the dideoxy chain termination method (Sanger 1977).

*In situ hybridization.* In situ hybridization to polytene chromosomes from salivary glands were carried out with *D. hydei* wildtype according to Vogt *et al.* (1986). Incubation conditions were 2 x SSC at 62°C for 6 h. Washing was with 2 x SSC for 1.5 h. We used nick-translated insert $^3$H-DNA of the clone mDh3VT2 as a probe. This insert DNA was obtained as described in chapter IV. Photographs were taken on Agfaortho 25 film with a Zeiss Photomicroscope III after Giemsa-staining.

*Analysis of Northern blots.* Isolation of RNA from testis and carcass, electrophoresis and hybridization were performed as described in chapter IV.

## Results

### *Cloning of an H3 histone variant cDNA*

From Northern blot hybridizations (chapter IV) we had strong indications for the existence of mRNAs coding for variants of different histones which in part seemed to be testis-specific. Because of the weak hybridization signals on some of these RNAs which suggests low amounts, we decided to amplify the cDNAs by the polymerase chain reaction (PCR) before cloning. Variant transcripts detected with a histone H3-specific probe gave the strongest signals on Northern blots and, therefore, were choosen to start with.

Total RNA of testis or carcass was reverse transcribed into a cDNA(-) strand with two different oligonucleotides, a $(dT)_{17}$ - primer (fig. 1A) and H3- primer I (Fig. 1B) derived from the sequence of the 3' end of the histone H3 gene present in pDhH6/7. This clone contains the genes coding for the presumptive cell-cycle regulated histones of *D. hydei* (Kremer and Hennig 1990). The H3-primer I was selected such that it contains the lowest possible sequence similarity with other histone sequences in the histone gene cluster.

The primer-extended products were separated from excess primer and subsequently a poly(dC) tail was added (Fig. 1C,D).

Synthesis of the second strand of the cDNA was carried out with the $(dG)_{12}$-adaptor primer. Finally, the cDNA was amplified with the adaptor primer and H3-primer II consisting of a sequence immediately upstream of the DNA stretch represented in H3-primer I (Fig. 1E).

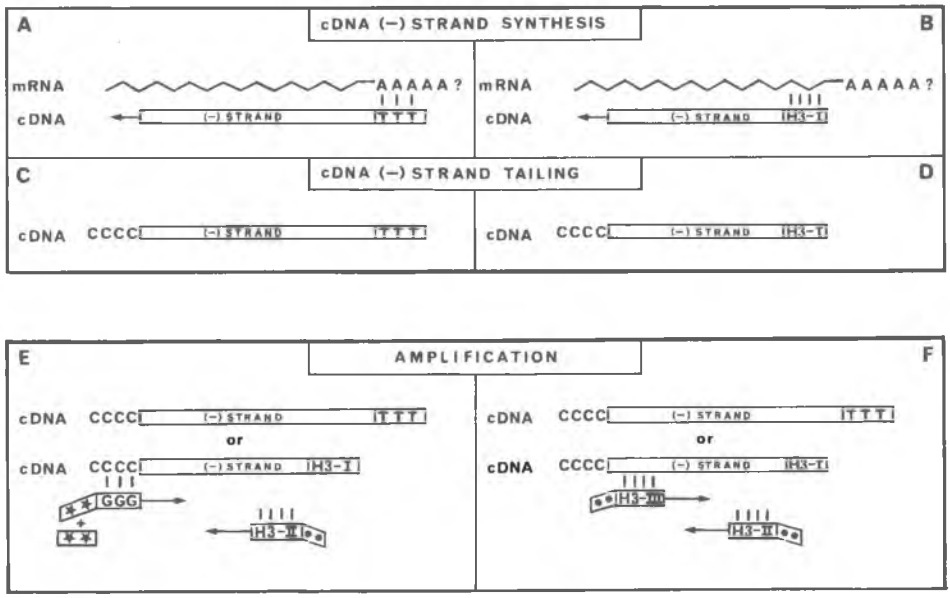


**Fig. 1.** Schematic representation of the procedure followed to amplify histone H3 variant cDNAs. The following primers were used: TIT , 5`- $(dT)_{17}$ -3`; H3-I 5` - CCTTGGGCATGATTGTGACA/G/C/TC - 3` (nt 2221-2241 in clone pDhH6/7); ⊙⊙ 5`- GCAGCGGCCGC - 3`, this sequence contains a Not I recognition site; H3-II 5`- GCAGTTGGCCACTAAGGCAGC - 3` (nt 2514-2534 in pDhH6/7); H3-III 5`- AATGG-CACACAAGTTTGTA7GTC - 3` (nt 2252-2272 in pDhH6/7); ★★ 5`- AGCTCTA-GAGCGGCCGCAAGCTT - 3`, this sequence contains a XbaI, Not I and Hind III recognition site, GGG , $(dG)_{12}$.

An alternative way of second strand synthesis and amplification of cDNAs was chosen where the $(dG)_{12}$-adaptor primer was replaced by H3-primer III. This primer is derived from the sequence of the 5' end of the pDhH6/7 H3 gene. In this way an internal region of the cDNA(-) strand was amplified (Fig. 1F). In figure 2 the described procedure is summarized.

The amplification products were analysed by gel electrophoresis, Southern blotting and hybridization with a $^{32}P$-labelled 21 nt long oligonucleotide sequence deduced from the H3 gene present in pDhH6/7 (nt 2371-2391). The PCR products that hybridized with the oligonucleotide probe had a length corresponding to that expected to arise from the pDhH6/7- derived H3 transcript, i.e. a length which is expected from the presumptive cell-cycle regulated transcript as template.

One of the amplified DNA fragments represents the internal region of the H3 gene (Fig. 1F). The cDNA(-) strands made with either the $(dT)_{17}$ -primer or with the H3 primer I (Fig. 1A,B) gave a strongly stained band at about 300 bp on ethidium bromide-stained gels after amplification with the H3 primers II and III (Fig. 1F). Hybridization experiments indicated that sequence variation may exist among these amplification products. If hybridized with the 21 nt H3-specific oligonucleotide a relatively faint signal was obtained for the $(dT)_{17}$ -cDNA derived products. This was found with testis as well as with carcass RNA as starting material.

The 300 bp DNA fragments from $(dT)_{17}$ -primed cDNAs representing both testis and carcass RNA were isolated from agarose gels and cloned into the vector M13 mp18. Four clones were isolated, three from testis RNA (designated as mDh3VT1, mDh3VT2 and mDh3VT3) and one from carcass RNA (mDh3VC1).

*Sequence analysis of the cDNA clones*

Figure 3 shows the nucleic acid sequences and figure 4 the deduced amino acid sequences of the three mDh3VT clones and of the clone mDh3VC1 in comparison to those of the H3 gene of pDhH6/7 (nucleotides 2513-2273, Kremer and Hennig 1990).

The clones mDh3VT1 and mDh3VT2 are clearly divergent in their nucleotide sequence from the corresponding region of pDhH6/7.

For the mDh3VT1-derived amino acid sequence five substitutions (pos. 6, 20, 62, 64, 65 ) exist in comparison with the pDhH6/7-derived H3 amino acid sequence. In the mDh3VT2-derived amino acid sequence only four of these substitutions (in positions 6, 62, 64 and 65) are seen. The substitutions at positions 6, 20, 62 and 64 are conservative ones.

In contrast to the sequence of the clones mDh3VT1 and mDh3VT2 the nucleotide sequence of the clone mDh3VT3 is identical to the sequence of the corresponding region of pDhH6/7 from nucleotide number 2 to 217. At position 1 there is a base substitution.

The mDh3VC1-derived amino acid sequence again differs at the positions 6, 53, 62, 64 and 65 from the pDhH6/7-derived H3 amino acid sequence.

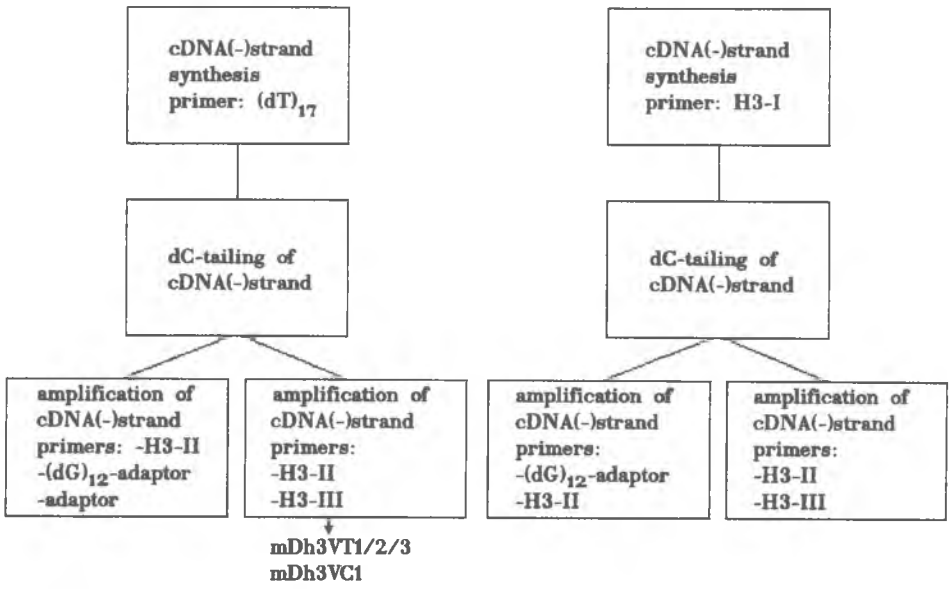


**Fig. 2.** Schematic representation of the procedure followed to amplify histone H3 variant messengers.

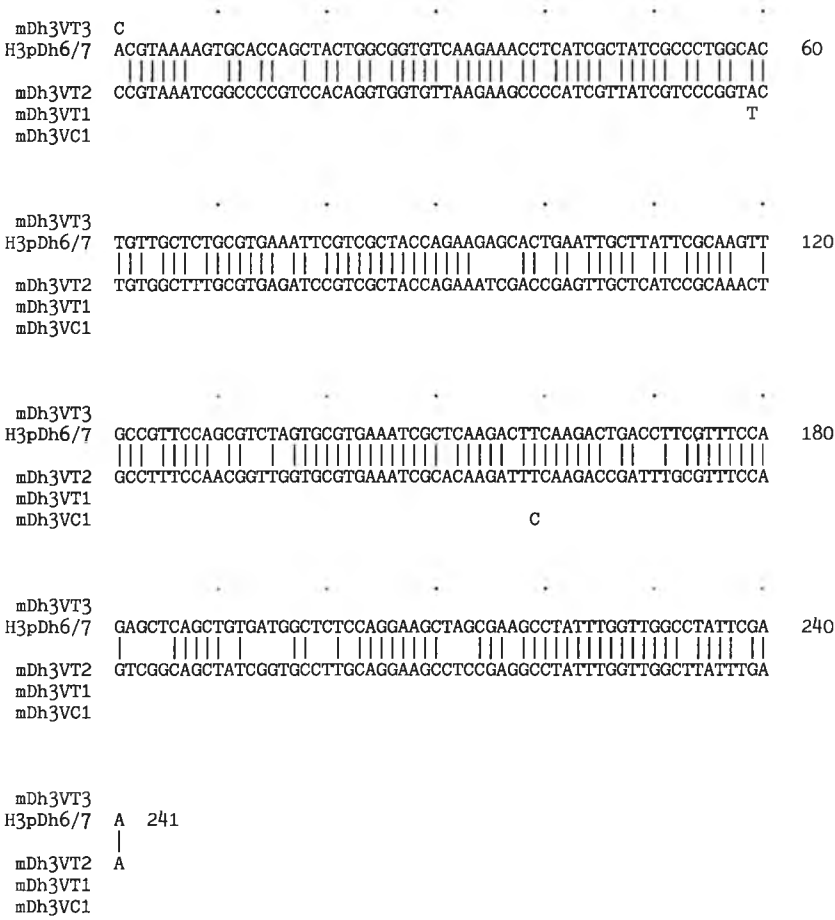


**Fig. 3.** Comparison of the nucleotide sequences of the clone mDh3VT2 with the corresponding region of pDhH6/7. mDh3VT2 represents the H3.3-like sequences, pDhH6/7 represents the putative cell-cycle regulated H3 gene. From the clone mDh3VT3 only the nucleotides deviating from pDhH6/7 are given. From the clones mDh3VT1 and mDh3VC1 only the nucleotides deviating from Dh3VT2 are shown.

### *Transcript analysis with the clone mDh3VT2 as a probe*

An obvious question is which of the poly(A)$^+$ RNAs seen after hybridization with a H3-specific probe (chapter IV), can be assigned to the described variant H3 clones. We hybridized nick-translated amplified insert DNA of mDh3 VT2 to Northern blots of testis and carcass poly(A)$^+$ and poly(A)$^-$ RNA. Washing was done under stringent conditions (0.02 M $Na_2HP0_4$, 50°C). Of the variant RNAs detected in testis poly(A)$^+$ RNA with the H3-specific probe (chapter IV, Fig. 3) only that of ~1900 nt and the heterogeneous fraction of ~750-1100 nt hybridized to mDh3VT2 (Fig. 5). The ~1900 poly(A)$^+$ RNA is also detected in carcass poly(A)$^+$ RNA. Here the signal is more intense than with testis poly(A)$^+$ RNA. In carcass RNA the region corresponding to RNA of ~800-1100 nt hybridizes only very faintly to the probe. Hardly any hybridization is detectable in poly(A)$^-$ RNA fractions from testes or carcass.

```
mDh3VT3
H3pDh6/7  RKSAPATGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQ   60
mDh3VT2        S
mDh3VT1        S              S
mDh3VC1        S                                                  S


mDh3VT3
H3pDh6/7  SSAVMALQEASEAYLVGLFE  80
mDh3VT2    A IG
mDh3VT1    A IG
mDh3VC1    A IG
```

**Fig. 4.** Comparison of the deduced amino acid sequences of the RNA derived clones with the deduced amino acid sequence of the corresponding region of pDhH6/7. Only the deviating amino acids are shown. The substitutions at the amino acids 6, 62, 64 and 65 are diagnostic for the H3.3 variant (Wells and McBride 1989).

### *Comparison of the cDNA amino acid sequences with known H3 primary sequence variants*

We compared the amino acid sequences coded for by mDh3VT1, mDh3VT2 and mDh3VC1, with amino acid sequences of H3 genes as compiled by Wells (1986) and Wells and McBride (1989). It turned out that the substitutions found at positions 6, 62, 64, and 65 (fig. 4) are diagnostic for H3.3 variants found in vertebrates. It has been described above that the amino acid sequences deduced from the DNA sequences of the clones mDh3VT1 and mDh3VC1 each show one additional substitution, mDh3VT1 in amino acid 20 (Ser instead of Thr) and mDh3VC1 in position 53 (Ser instead of Phe). These substitutions are not shared by any of the sequences of the H3.3 variants given by Wells (1986) and Wells and McBride (1989).

### *Comparison of H3 coding sequences*

The DNA sequences of the variant mDh3VT1 (or mDh3VT2 and mDh3VC1) and the corresponding region of the *D. hydei* presumptive cell-cycle regulated H3 gene (in pDh6/7) (Kremer and Hennig, 1990) are identical for 76%. The percen-


**Fig. 5.** Northern blot hybridizations with nick-translated insert DNA of mDh3VT2 as a probe on the poly(A)$^+$ and poly(A)$^-$ fractions of testis and carcass RNA. About 1 μg of poly(A)$^+$ RNA and about 20 μg of poly(A)$^-$ RNA were loaded. Stringent washing conditions (0,02 M $Na_2HPO_4$, 50°C) were used. The size markers were from BRL (1.77-0.16 kb). The lengths of the transcripts are given in nucleotides.t, testis; c, carcass; A$^+$, poly(A)$^+$ fraction; A$^-$, poly(A)$^-$ fraction.

tage of similarity between the latter DNA sequence and the corresponding region of the presumptive cell-cycle regulated H3 gene of *D. melanogaster* (Matsuo and Yamazaki 1989) is about 83%. Thus the degree of DNA sequence similarity between the presumptive cell-cycle regulated H3 genes of the two different species is higher than between mDh3VT2 and the presumptive cell-cycle regulated H3 gene of *D. hydei*.

### *In situ hybridization*

*In situ* hybridization on polytene chromosomes was performed using insert DNA of mDh3VT2 as a probe. Signals were found in three different regions: region 10C of the X chromosome, region 62A2/3 of chromosome 3, and region 116B/C of chromosome 5 (fig. 6). Hybridization on chromosome 3 might be the result of a weak cross-hybridization to the cluster of presumptive cell-cycle regulated histone genes.

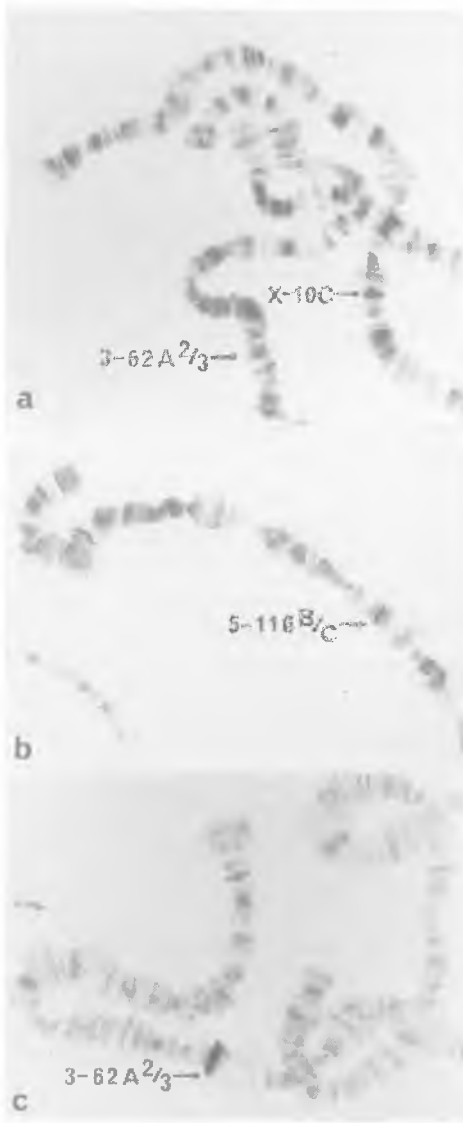


**Fig. 6.** *In situ* **hybridization on polytene chromsomes of *Drosophila hydei* with $^{3}$H-labeled mDhVT2 (a,b) or mDhH2B (c) as a probe. mDhH2B is a representative of the cell-cycle regulated histone genes (see Fig. 1, chapter IV). With the latter probe only a signal on the main histone gene cluster is observed.**

## Discussion

Studies of histone transcripts in testis and carcass RNA suggested that histone variants exist in *D. hydei* (chapter IV). Some of them seemed - in contrast to the common histones - to be translated from polyadenylated RNA species. In this paper we described four cloned cDNA fragments which were obtained from testis and carcass poly(A)$^{+}$ RNA after PCR amplification. Sequencing revealed three partly different nucleotide sequences, two derived from testis RNA and the third from carcass RNA. The deduced amino acid sequences for each of these fragments displayed either

close similarity or identity with the H3.3 variants. The H3.3 variants were originally described for vertebrates (see Wells 1986; Wells and McBridge 1989) but subsequently found in molluscs (Swenson *et al.* 1987). The indications for the presence of H3.3 coding sequences in the genome of *Drosophila* further supports the idea promoted by Wells and coworkers (Wells *et al.* 1986) that H3.3 genes are ancient constituents of eukaryotic genomes.

H3.3 variants are specifically characterized by a Ser in position 31, Ala in position 87, Ileu in position 89 and Gly in position 90 (*cf.* fig. 4). The positions mentioned correspond to amino acid positions 6, 62, 64 and 65 respectively of the *Drosophila* histone H3 in figure 4. All these substitutions occur in the deduced amino acid sequence of clone mDh3VT2 of *D. hydei*. The sequence of the two clones mDhVT1 and mDhVC1 indicate different additional amino acid substitutions. The deduced amino acid sequence of clone mDhVT1 has a Ser in position 45, that of clone mDh3VC1 a Ser in position 78, corresponding to positions 20 and 53 respectively in figure 4. Both additional amino acid substitution are not contained in any of the H3 or H3.3 variant histones compiled by Wells (1986) and Wells and McBridge (1989).

At present we cannot exclude that the differences between clones mDhVT1, mDh3VT2 and mDh3VC1 are the result of an artefact during the PCR amplification. In addition, it cannot with certainty be concluded that the clones derived from testis RNA are derived from germ cells since the testes contain substantial numbers of somatic cells (Grond 1984; for review see Hennig and Kremer 1990), nor can it be excluded that the clone obtained from carcass RNA originates from a germ cell, although contamination of carcass with residual testis material is less likely (see below).

However, it can be excluded that the clones obtained are derived from small contaminations with genomic DNA in the poly(A)$^+$ RNA which may lead to an amplification of DNA sequences of a nontranscribed pseudogene, present in the genome as a relict of the H3.3 family (*cf.* Wells *et al.* 1986). The cloned DNA hybridizes to testis and carcass RNA fractions different in length from the universal H3 (poly(A)$^-$) RNA fraction (Fig. 5). These observations, together with the only very low level of cross-hybridization with the presumptive cell-cycle regulated H3 RNA species, demonstrate the expression of at least one H3.3 variant histone gene in *Drosophila*. Additional support to this conclusion is provided by the results of *in situ* hybridization on polytene chromosomes which revealed two different genomic locations of H3.3 DNA sequences outside the main cluster. The hybridization signal on the main cluster indicates either H3.3 sequences in or very close to it. On the other hand this signal might be due to a weak cross reaction of the H3.3 probe to the presumptive cell-cycle regulated H3 genes. Analysis of Southern blots of genomic DNA with mDhVT2 as a probe so far gave no information for the existence of H3.3 genes outside the main cluster. This might be due to the strength of the signal derived from the main cluster, which prevents identification of additional minor band.

Since relative differences exist between the hybridization of the various RNA species in carcass and testis, it is also clear that the expression of H3.3 variant histone(s) differs between somatic cells and testis. The relatively strong signals obtained in testis RNA make it, in addition, unlikely that this RNA is derived from the somatic part of the gonad since the relative contribution of RNA from the somatic testis cells is small (Hennig 1967). It is, thus, likely that H3.3

variant histones are expressed in germ cells and play a role in the chromatin organization of meiotic or postmeiotic chromosomes in the male.

The clone mDh3VT3, identical to the corresponding region of pDHh6/7, might have originated from a polyadenylated transcript from an H3 gene primarily involved in cell-cycle regulated transcription. Polyadenylated transcripts can be synthesized through the use of an alternative pathway in the 3′ end formation as has been shown for an H2A and an H2B gene in avian haploid round spermatids (Challoner *et al.* 1989). Such transcripts might be represented in the ~1400 nt H3 transcripts (Table 2, Chapter IV).

### *The histone H3.3 gene of Drosophila is member of a multigene family*

The three cDNA clones mDh3VT1, mDh3VT2 and mDh3VC1 isolated from testis and carcass poly(A)$^+$ RNA suggest that H3.3 histone variants are a universal feature of the genome of higher eukaryotes. The differences in the nucleotide sequences of the three clones suggest that they are not derived from a single gene but represent transcripts of different gene copies, although one might argue that the differences are artefacts of the PCR reaction. The demonstration of H3.3.-related DNA sequences in at least two different genomic positions (X chromosome region 10C, chromosome 5 region 116B/C) of *D. hydei* by in situ hybridization to polytene chromosomes makes it, however, most likely that more than one functional gene of the H3.3 family is present in the genome (see also page 11).

A histone H3.3 multigene family with 20-30 members has been found in the human genome. Several of these multiple gene sequences were shown to be processed pseudogenes. It seems that only one or a few functional H3.3 genes exist since all 20 of the cDNAs isolated from a fibroblast cDNA library appear to be encoded by the same gene (Wells and Kedes 1985; Wells *et al.* 1987). Also for chicken only two functional H3.3 genes have been described, which code for two identical protein chains (Brush *et al.* 1985). But even this argument does not exclude the possibility that multiple identical gene copies of the H3.3 variant exist within the H3.3 multigene family.

### *Multiple transcripts of one gene ?*

In Northern blots, mDh3VT2 hybridizes under stringent washing conditions to poly(A)$^+$ RNA from testis and carcass. The RNA species detected (~1900 nt and the heterogeneous RNA of ~900 nt) are longer than expected for a H3 histone mRNA. Similar observations of an increased length of the H3.3 variant mRNA have, however, been made for the mouse (Hraba-Renevey and Kress 1989), the chicken (Brush *et al.* 1985) and for man (Wells and Kedes 1985).

What is the origin of two length types of RNA and what is the reason for the increased lengths of the transcripts in *D. hydei* ? An explanation for the existence of two length classes of transcripts is that they are transcribed from different genes. The cloning of two only slightly differing fragments (mDh3VT1 and mDh3VT2) supports this assumption. However, in mouse kidney culture cells H3.3 mRNAs with three different lenghts (1.8, 1.2 and 1.0 kb) are found which most probably result from one gene through the use of different polyadenylation signals (Hrabe-Renevey and Kress (1989). The same explanation can be applied

to the ~1900 nt and ~900 nt H3 poly(A)$^+$ RNAs found in testis RNA. The polyadenylation signal responsible for the production of the ~900 nt RNA might be preferentially used in testis while the polyadenylation signal responsible for the production of the ~1900 nt RNA might be used to a minor extent in testis. In carcass tissue the use of the latter polyadenylation signal might be the preferential way to produce an H3.3 mRNA.

The existence of H3.3 variant mRNAs of two different lengths in testis poly(A)$^+$ RNA may also be explained in another way. Introns, generally present in genes coding for replacement histone variants (*cf* Brush *et al.* 1985; van Daal *et al.* 1990) might be spliced out only late during (transcriptionally inactive) postmeiotic stages of spermatogenesis. Since splicing is a prerequisite for translation, a regulatory mechanism for translation of this mRNA may be created in this way (*cf.* germ-line-specific splicing of the P-element ORF2-ORF3 intron (Laski and Rubin 1989)). By a low efficiency of splicing of the ~1900 nt mRNA in carcass the corresponding gene might become preferentially expressed in the testis.

*Codon usage of histone genes in Drosophila is atypical.*

Wells *et al.* (1986) showed for H3 genes of vertebrates that the codon usage pattern is correlated to the function of the gene: (presumptive) cell-cycle regulated H3 genes have a bias for G or C in the third codon positions which is not true for the replacement variant H3.3 genes.

We compared the available part of the sequence of a presumptive H3.3 variant of *D. hydei* with the corresponding part of the presumptive cell-cycle regulated H3 genes of both *D. hydei* and *D. melanogaster*. We found no preference for G or C in the third codon position in both the presumptive cell-cycle regulated H3 genes or the presumptive H3.3. coding region. The G+C content in the third codon position in the clones mdDh3VT1, mDh3VT2 and mDh3VC1 is about 56%. In the corresponding regions of the presumptive cell-cycle regulated H3 genes of *D. hydei* and *D. melanogaster* this is 45% and 62% respectively. These values do not change significantly for the total H3 coding regions of the presumptive cell-cycle regulated genes.

Shields *et al.* (1988) argue that the heterogeneity in codon usage among *D. melanogaster* "cannot be interpreted simply as the result of mutational biases but also reflects the action of natural selection". They showed that for *D. melanogaster* genes in general, codon usage bias is associated with a change in base composition at silent sites. Furthermore there seemed to be a relationship between the level of gene expression and the strength of codon usage bias.

Extrapolating this conclusion to the histone genes of *Drosophila*, it would mean that both the presumptive cell-cycle regulated histone genes and the presumptive H3.3 gene do not belong to the highly expressed genes. This is unexpected for the presumptive cell-cycle regulated genes which are highly expressed in *Drosophila* early embryos with their high rate of cell divisions (Zalokar and Erk 1976). Is a relatively low efficiency of translation of histone mRNAs in early embryos compensated by a particularly high abundance of histone messenger RNA or does the tRNA population of early embryos deviate from that of other cell types by being adapted to a random codon usage ?

*Concluding remarks.*

Wells *et al.* (1986) proposed a model predicting that the H3.3 class of H3 genes is the most ancient one and that H3.3-like genes are ubiquitous, although they had not been detected in invertebrates. Since then, a cDNA coding for an H3.3 variant histone protein from the clam *Spisula solidissima*, has been described (Swenson *et al.* 1987).

The sequences of the clones mDh3VT1, mDh3VT2 and mDh3VC1 give the first indications for the existence of a H3.3-like replacement variant in *Drosophila*. The difference at isocoding positions between the sequence of the clone mDh3VT2 and the corresponding region of the H3 gene of the clone pDhH6/7 approaches maximal random divergence. This is in agreement with a very ancient origin of both sequences.

Little is known about the function of replacement variants. The amino acid sequence of the H3.3 variants and the H2A variants of the H2A.F/Z type, from which the H2AvD variant of *D. melanogaster* is a representative (van Daal *et al.* 1988), are highly conserved in evolution (Wells 1986; Wells and McBride 1989; Wu *et al.* 1982a). This indicates an essential role of these proteins. In their patterns of synthesis during the cell-cycle they differ from their cell-cycle regulated counterparts (Hraba-Renevey and Kress 1989; Wu *et al.* 1982b). In vertebrates variants are synthesized in several cell-types, in embryos as well as in differentiated tissues (Brush *et al.* 1985; Wu and Bonner 1981; Wu *et al.* 1982b; Wu *et al.* 1983). In clam oocytes H3.3 variant transcripts are stored to be translated after fertilization (Swenson *et al.* 1987). Whether the variant is specific for embryogenesis is unknown. For the H2A.F/Z-homologous variants it is suggested that they are preferentially associated with transcriptionally active chromatin (reviewed by Gorovsky 1985).

These observations indicate a general function for replacement histone variants. Whether this is also true for *Drosophila* remains to be established since we have no information yet on the expression of H3.3 in specific cell-types of the testis or during specific stages of spermatogenesis. In *D. hydei* a gene coding for an H3.3-like histone variant either might use a testis-specific 3' end or it might have evolved into a gene which is preferentially expressed in testis. The specificity of expression might be mediated by testis-specific splicing of the ~1900 nt transcripts as discussed above.

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## CONCLUSIONS AND SUMMARY

The studies described in this thesis address the question of chromatin reorganization during spermatogenesis of *D. hydei*. From the lightmicroscopy of DAPI-stained developing male germ cells two main remarkable points regarding the chromatin condensation emerged (chapter II):

1. The condensation of the chromosomes during the meiotic prophase I is late. The decondensed state of the chromatin is maintained after transcription has decreased to a level below radiographic detectability after incorporation of $^{3}$ H-labeled uridine (Hennig, 1967).
2. There is a cycle of condensation and subsequent decondensation of the chromatin in early spermiogenic cells. This is accompanied by changes in the number and structure of the protein bodies present in the nucleus of early spermatids.

The analysis of the expression of histone genes was started after characterization of a representative of the main type of histone gene cluster, which codes for the presumptive cell-cycle regulated histones (chapter III). In both testis and carcass, histone messenger RNA species were detected, which differ from the nonpolyadenylated messengers coding for the presumptive cell-cycle regulated histones. Differences in length and in sequence (as can be deduced from the hybridization signals using different washing conditions) indicate the existence of testis-specific variants for the histones H2B, H3 and possibly for H4 and H1, but not for H2A (Chapter IV).

cDNA cloning of variant H3 messengers indicated the presence of an H3.3 like histone in both germ cells and somatic tissues. The amino acid substitutions indicated by the nucleotid sequence of the available part of cDNAs are diagnostic for this H3 variant (Chapter V).

Can the cytological observations be correlated to the molecular data? For histone H1 and H2A cytological data are confirmed by the transcript analysis. The absence of H1 in the chromatin as indicated by the lack of detectable immunofluorescence from the early primary spermatocyte stage on coincides with the low amount of messengers coding for the presumptive cell-cycle regulated H1, compared to the amount of messengers coding for the core histones. Histone H2A, on the other hand, was clearly detected immunocytochemically in male germ cells up to late elongating spermatids. This finds its correlation in the relatively high amount of the corresponding presumptive cell-cycle regulated messengers and the low detectable amount of variant H2A messengers.

So far, however, we cannot answer the question on the presence of histone variants in male germ cells and the involvement of these variants in the cytologically observed chromatin reorganizations. For the histones H2A, H2B, and H1 either no testis-specific variant messengers, or only very low amounts, were detectable. This is remarkable since these histones display the highest degree of variation within organisms, both in male germ cells and in somatic cells. However, the fact that variant messengers of these histones are not detectable, or give only weak signals in hybridization analysis, does not mean that they are absent or present in a low amount. They might (nearly) have escaped detection because of a low sequence similarity to DNA of the presumptive cell-cycle regulated genes which were used as a probe in the transcript analysis. In addition, their messengers might not be detectable because they lack a poly(A)$^{+}$ tail and

may have the same length as those coding for cell-cycle regulated histones. For the histones H3 and H4 presence of (testis-specific) variant histone messengers in the testis is more clearly indicated. The intensity of the hybridization signals of the variant messengers in testis RNA makes a somatic origin very unlikely since, the somatic cells in terms of volume are only a minor part of the testis-tissue.

To prove the involvement of histone variants in chromatin reorganization during spermatogenesis the functionality of the variant histone messengers and their germ line-origin has to be demonstrated. So far we have only cDNA sequence data for a variant H3 messenger. Although the cloned cDNAs are not full length, the uninterrupted open reading frame, together with the identity of the predicted amino acid sequence of one of the cDNAs with a conserved H3 variant, H3.3 in vertebrates, indicate functionality of these messengers.

As discussed in the introduction speculation on the function of histone variants during spermatogenesis is very difficult. Our studies did not result in new arguments for this discussion, especially because of the absence of information on the cell types in which the putative variants are expressed. However, it is most likely that the putative histone variants are deposited in the chromatin before its final condensation late during spermiogenesis. Coincident with this condensation a substitution of histones by arginine-rich proteins can be assumed, since this is indicated to occur during *D. melanogaster* spermiogenesis (Das *et al.* 1964; Hauschteck-Jungen and Hartl 1982). This extrapolation can be made because spermatogenesis of *D. hydei* closely resembles that of *D. melanogaster* (reviewed by Hennig and Kremer 1990). The putative testis-specific H1 variant might have its function in the organization of the chromatin during meiotic prophase and in this way be involved in transcriptional inactivation of the chromatin. The immunofluorescence data indicate through the lack of reaction with the antibody against somatic H1 an occurrence of this variant from the early primary spermatocyte stage on. For the putative variants of the histones H2B, H3 and H4 we have no indications for the time of transcription and/or translation of their messengers. As one alternative these histone variants may occur in the chromatin immediately before or during the meiotic prophase with the extended period of decondensed chromatin. These are the stages where histone variants are deposited in the male germ cell chromatin in mammals and sea urchins (chapter I).

An alternative stage for deposition of putative histone variants in the chromatin is early spermiogenesis where a complex rearrangement of the chromatin occurs. In males with a completely or partly deleted Y chromosome these early postmeiotic changes in chromatin organization are absent or incomplete, whereas chromatin organization in spermatogonia and primary spermatocytes is comparable to wild-type males as far as can be observed by lightmicroscopy (Kremer *et al.* unpublished). Investigation of the variant histone messengers and/or the putative corresponding proteins in the testes of these mutant males can answer the question whether the putative histone variants are involved in the early postmeiotic chromatin reorganization or whether they have their specific function before or after early spermiogenesis (*cf.* ssH2B in mouse spermatids, Moss *et al.* 1989).

The cDNA sequences isolated for the H3.3 histone variant allow a more concrete speculation on its function. As in vertebrates the H3.3 variant of *Drosophila hydei* seems to be generally expressed in a variety of tissues

(Brand and Kremer unpublished). In male germ cells, however, this variant might have a special function. In man about 15% of the DNA of mature sperm, probably a sequence-specific subset, remains associated with histones including the H3.3 variant (Gatewood *et al.* 1990). These authors suggest that the nucleohistone of the sperm may represent chromatin which is programmed for early development due to the association with specific histone variants and specific transcription factors. The putative expression of histone H3.3 in *Drosophila hydei* male germ cells suggest that the phenomenon described for human sperm might be a general one.

To get further insight into the diversity of histone variants in *Drosophila* spermatogenesis and their influence in chromatin function during that process cDNA clones of the variant messengers have to be isolated. As can be deduced from the transcript analysis (Chapter IV) part of these histone variants are expected to be specific for the testis. Especially these variants are suitable for studies on the function of testis-specific histone variants since mutation of the corresponding genes will not be lethal. For the isolation of specific mutants *D. melanogaster* is the highly preferred species. The possibility of site-selected transposon mutagenesis for this organism makes it relatively easy to isolate P-element-induced alleles of specific genes (Kaiser and Goodwin 1990). Regarding the evolutionary conservation of histones and the similarity of spermatogenesis in both species, the switch to *D. melanogaster* as study object must be easy to make. With the results of the studies described in this thesis we expect it to be possible to analyse the function of testis-specific histone variants in the near future.

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## SAMENVATTING

Spermatogenese is een ontwikkelings-proces waarbij zich uit een "ongedifferentieerde" kiemcel een hoog gedifferentieerde zaadcel ontwikkelt. Het functioneren van het chromatine is een van de meest fascinerende aspecten van deze ontwikkeling. Behalve een differentiele gen-expressie die de basis vormt voor de differentiatie van de cellulaire structuren, karakteristiek voor een zaadcel, zien we in de meeste organismen een ingrijpende verandering in de organisatie van het DNA in de celkern. Bovendien is in de afgelopen jaren steeds duidelijker geworden dat er tijdens de zaadcelvorming een markering van het genoom plaats vindt die essentieel is voor de verdere ontwikkeling van een bevruchte eicel. Bij het in dit proefschrift beschreven onderzoek werd het aspect van de reorganisatie van het chromatine nader bekeken bij de fruitvlieg *Drosophila hydei*. Allereerst werd een cytologische analyse van deze reorganisatie uitgevoerd na kleuring van het DNA met DAPI (4′,6-damidino-2-phenylindole dichloride). Daarbij werd een complexe reorganisatie van het chromatine aangetoond. Het proces volgend was allereerst de condensatie van de chromosomen zichtbaar die gepaard gaat met de meiose. Opvallend hierbij was het late tijdstip van de condensatie tijdens profase I. Meteen volgend op de meiose werd een condensatie van het chromatine waargenomen, gevolgd door een decondensatie tijdens het begin van de elongatie van de zich ontwikkelende zaadcel. Een dergelijke reorganisatie werd, voor zover ons bekend, niet eerder beschreven. Volgend op de elongatie-fase van de zaadcelontwikkeling werd de uiteindelijke condensatie van het chromatine aangetoond. Immunocytochemische pilot-studies gaven voor histon H1 al een vroege verandering aan.

Om het onderzoek aan de chromatine reorganisatie, en meer in het bijzonder aan de rol van histonen hierin, op moleculair niveau voort te zetten, werden allereerst de "normale" histon genen die cel-cyclus afhankelijk gereguleerd worden, geanalyseerd. Daarbij werd gevonden dat de promoter elementen die bij eukaryoten verantwoordelijk uijn voor de cel-cyclus afhankelijke regulatie van de transcriptie, bij de *Drosophila* histon genen ontbreken.

Met de cel-cyclus afhankelijk gereguleerde histon genen als sonde werd de transcriptie van histon genen in de testis bekeken. Ter vergelijking werd ook RNA geanalyseerd van mannetjes waaruit de testes verwijderd waren. Er werden 3 typen histon transcripten gevonden die afweken van de "normale" histon transcripten.

I. Transcripten die alleen in lengte afwijken van de "normale" histon transcripten.
II. Transcripten die zowel in lengte afwijken als ook in de polyadenylatie. In tegenstelling tot de cel-cyclus afhankelijk gereguleerde histon transcripten, komen histon messengers van dit type voor in de fractie van gepolyadenyleerd RNA.
III. Transcripten die niet alleen in lengte en polyadenylatie afwijken van hun cel-cyclus gereguleerde tegenhangers, maar ook in DNA sequentie.

Messenger RNAs van dit laatste type geven aan dat er mogelijk histon varianten voorkomen die tijdens de spermatogenese normale histonen geheel of gedeeltelijk vervangen. DNA sequentie bepaling van een copie DNA van een van de type III

histon H3 messengers liet zien dat deze waarschijnlijk codeert voor een H3.3 variant eerder beschreven voor vertebraten en een mossel. Mogelijk heeft deze variant en specifieke functie tijdens de spermatogenese.

Het voorkomen van de type III variant messengers die ten dele specifiek zijn voor de testis, biedt, na verdere karakterisatie van deze messengers, goede mogelijkheden voor verder onderzoek. Met name voor onderzoek naar de functie van testis-specifieke histon varianten is *Drosophila* een uitstekend model systeem. De mogelijkheden die er met name bij *Drosophila melanogaster* zijn voor het maken en analyseren van mutanten, bieden mogelijkheden die tot dusver ontbraken voor onderzoek naar de functie van testis-specifieke histon varianten.

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Hannie

# *CURRICULUM VITAE*

Hannie Kremer werd geboren te Heerlen op 6 juli 1959. Zij volgde de Atheneum opleiding aan het "Sophianum" te Vaals 1971 tot 1977. Aansluitend begon zij haar studie Biologie aan de Katholieke Universiteit Nijmegen. Het doctoraal examen legde zij in 1984 af met als hoofdvak microbiologie (Prof. Vogels) en als bijvakken geobotanie (Prof. Westhoff) en genetica (Prof. Hennig). Tevens werd de onderwijsbevoegdheid 1$^{e}$ graads behaald. Van september 1984 tot september 1990 was zij als wetenschappelijk medewerker verbonden aan de vakgroep moleculaire en ontwikkelingsgenetica van de Katholieke Universiteit Nijmegen. Hier werd o.l.v. Prof. Dr. W. Hennig het in dit proefschrift beschreven promotie onderzoek uitgevoerd. Zij was tijdens deze periode betrokken bij het onderwijs aan biologiestudenten. Sinds december 1990 is zij wetenschappelijk onderzoeker bij het instituut voor Antropogenetica van de Katholieke Universiteit.